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Subsurface Igneous Mineral Microbiology: Iron-Oxidizing Organotrophs on Olivine Surfaces and the Significance of Mineral Heterogeneity in Basalts

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by

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A thesis submitted in partial fulfillment of the
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ABSTRACT

The subsurface igneous biome contains a vast portion of Earth's total biomass, yet we still know so little about it. Igneous environments such as iron-rich ocean crust and lava tubes may also host analogs to chemolithotrophically-driven life on other planets, so studying life in this biome is essential to understanding how life may survive on other planets. In this study, three igneous surface and subsurface environments were investigated for microbial preference for olivine, microbial physiologies and phylotypes present on olivine, and microbial growth on olivine in the laboratory via iron oxidation. These environments include a subseafloor borehole drilled into the ocean crust basalt basement, a lava tube with perennial ice, and a trio of Columbia River basalt-hosted freshwater terrestrial habitats.

The subseafloor borehole (IODP Hole 1301A) is situated on the eastern flank of Juan de Fuca Ridge (JFR) and was used in the first long-term deployment of microbial enrichment flow cells using osmotically-driven pumps. The flow cells contained igneous minerals and glasses, for which cell density and microbial abundances were evaluated. Total cell density and viable oligotrophs were highest for Fe(II)-rich olivines. Organotrophic bacterial isolates were capapble of iron oxidation and nitrate reduction, and grew on olivine in the laboratory. Putative neutrophilic iron oxidizers were also isolated from igneous riparian and cave environments in northwest and central Oregon. Isolated bacteria from all three environments were capable of chemolithotrophic growth with olivine and oxygen or nitrate in the laboratory. Bacteria isolated from river basalt were putatively capable of producing alteration textures on olivine surfaces in culture.

Microbial life in the igneous subsurface preferentially attach to Fe^{2+}-rich minerals, which suggests that life in the subsurface is heterogeneously distributed. The isolation of oligotrophic iron oxidizers that grow on olivine suggests that olivine supports a chemolithotrophic subsurface community based on primary productivity via iron oxidation. This generation of biomass on olivine surfaces creates organic carbon-rich coated mineral surfaces that may support a more complex community. The identification of Mars analogs living in Oregon lava tubes and the discovery that iron oxidizers may produce biosignatures on olivine surfaces are key findings that may provide the foundation for a new chapter in the search for life on Mars.

DEDICATION

I would like to dedicate this work to my wonderful husband Shane and our own little science project, Stella PixieBelle.

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CHAPTER 1

INTRODUCTION

Olivine $(Mg,Fe)_2SiO_4$ is one of the most abundant minerals on Earth and is commonly found in mafic and ultramafic igneous rocks in both ocean crust and continental crust. The disequilibrium between oxidants and the Fe^{2+} from olivine can be utilized as a source of energy for microbial growth. However, there are only a handful of reports of microbes that were grown on or incubated with olivine in the laboratory [*Santelli et al*, 2001; *Welch and Banfield*, 2002; *Shirokova et al*., 2010; *Garcia et al*., 2005; *Longazo et al*., 2001, 2002; *Josef et al.*, 2007]. None of these studies used microbes that were isolated from olivine-bearing rocks, and only two reports (using the same species of bacteria, *Acidothiobacillus ferrooxidans*) from the same laboratory where olivine was used as the sole source of energy [*Santelli et al*, 2001; *Welch and Banfield*, 2002]. Little is known about olivine's microbiology, its role in supporting subsurface microbial populations, and it significance in terms of geochemical cycling of elements. Olivine comprises approximately 50% of the upper mantle, from which ocean crustal rocks are derived [*Deer et al*., 1992]. Olivine is also abundant in ocean crust, and olivine reactivity in subseafloor habitats may support a significant portion of the ocean crust biome. Olivine in aphotic, oligotrophic environments such as lava tubes or other deep subsurface habitats could provide the bulk of energy for primary productivity. In addition, olivine is universally abundant and understanding the microbiology of olivine on Earth will help us understand how life on other planets may use (or may have used) this mineral for energy.

Since basalts are mineralogically heterogeneous on a microscopic and macroscopic scale, endolithic populations that prefer olivine would be heterogeneously distributed in basalts and in the crust as a whole. Previous studies of subsurface basalt aquifer microbiology have primarily focused on unattached, free-living microbes in aquifer fluids or attached communities from whole pieces of rock that are mineralogically heterogeneous. In order to determine the true distribution of life underground, and ultimately calculate its role in geochemical cycling, mineral-focused studies must be undertaken. A major portion of this thesis is dedicated to the study of subsurface mineral microbiology in ocean crust. This study demonstrates that mineral heterogeneity in basalts influences the distribution of attached microbial populations in the subseafloor, and that olivine has the greatest abundance of cells when compared to other igneous minerals. Another chapter of this thesis is dedicated to the study of microbes in freshwater basalt-dominated systems that are grown on olivine in the laboratory, their metabolic properties, and mineral weathering textures they may produce. Finally, Chapter 5 describes the role of olivine in a Mars-analogue environment to gain an astrobiological perspective on microbe-mineral interactions.

General approach

This study focuses on endoliths in igneous rock since the majority of Earth's upper crust is mantle-derived igneous rock. A variety of environments are investigated, including deep ocean crust, terrestrial riparian environments and soils, and lava tube caves. This study focuses on enriching for iron oxidizers from multiple environments

and studying their growth on olivine in the laboratory. All isolates were identified by 16S rRNA gene sequencing and were subjected to phylogenetic analysis. A new approach for enriching microbes in the subsurface using monomineral substrates housed in flow cells connected to an osmotic pump was implemented to study the significance of mineral heterogeneity in basalts. Briefly, mineral weathering textures that may be produced by isolates in olivine media is explained. Finally, isolated microbes were tested for iron oxidation and nitrate reduction capabilities to help support the putative physiological capability of olivine oxidation.

Hypotheses

Five major hypotheses were drafted that apply to the study of the microbial ecology of olivine and the significance of mineral heterogeneity in basalts.

Hyp 1. Mineral heterogeneity influences microbial distributions in subseafloor basalts (*i.e.*, total microbial abundances will vary according to mineral).

Hyp 2. Energy-rich minerals (such as olivine) will host a greater abundance of life than energy-poor minerals.

Hyp 3. Community structure of attached microbes will vary according to source mineral (*i.e.*, iron-rich minerals will host different microbial communities than iron-poor minerals; iron oxidizers will be more prevalent on iron-rich mineral surfaces).

Hyp 4. Microbes attached to olivine in surface and subsurface environments are capable of iron oxidation using oxygen or nitrate as terminal electron acceptors.

Hyp 5. Subsurface psychrophilic iron-oxidizing microbes are found on Earth today and are physiologically capable of living on Mars today or in the past when liquid water was present.

Primary questions to address when testing the general hypotheses:

- What are the microbial colonization preferences for igneous minerals and glasses in the subseafloor?
- Are iron oxidizers living on olivine surfaces in nature?
- Can microbes isolated from a variety of sources (*i.e.*, soil, sediment, and basalt) be grown on olivine in the laboratory?
- Do psychrophilic iron oxidizers live in Mars-analogous environments such as ice lava tube basalt?
- Do microbes growing on olivine leave detectable biosignatures in the form of mineral weathering textures?

Specific Aims

1. Incubate a flow cell system containing a variety of igneous minerals and glasses in an ocean crust borehole for four years
2. Analyze recovered minerals to identify microbial colonization preference and phylogenetic diversity as they correlate to mineral composition
3. Determine if iron oxidizers and nitrate reducers are present on olivine in nature and study their growth on olivine in the laboratory

4. Isolate iron-oxidizing microbes from a variety of igneous-derived environments using olivine enrichments
5. Enrich for, identify, and study the physiology of psychrophilic iron oxidizers from lava tubes containing ice year-round

CHAPTER 2

REVIEW OF LITERATURE

Olivine

Olivine is a magnesium iron silicate [$(Mg,Fe)_2SiO_4$] that is common in ultramafic and mafic rocks such as peridotite and basalt. Olivine minerals crystallize very early (at 1900^o C – 1200^o C) from cooling mantle rocks [*Klein and Hurlburt*, 1985] (Figure 2.1), yet olivine is one of the fastest-weathering silicate minerals on Earth. The iron contained in the olivine crystal structure is only reduced iron (Fe^{2+}), although a coating of oxidized iron (Fe^{3+}) may exist under certain conditions. The disequilibrium between environmental oxidants and the Fe^{2+} from olivine can be utilized as a source of energy for microbial growth. Magnesium and silicon are both inert (not redox-active) and cannot be used for extracting energy. Olivine has varying amounts of Mg and Fe, depending on the conditions under which it was formed. Olivine is named based on the percentage of Mg to Fe within its structure. Forsterite is the Mg endmember (100% Mg), and fayalite is the Fe endmember (100% Fe). The designation for olivine is based on the ratio of magnesium to iron. For example, forsterite is labeled Fo_{100} (100% Mg, 0% Fe), and fayalite is Fo_0 (0% Mg, 100% Fe).

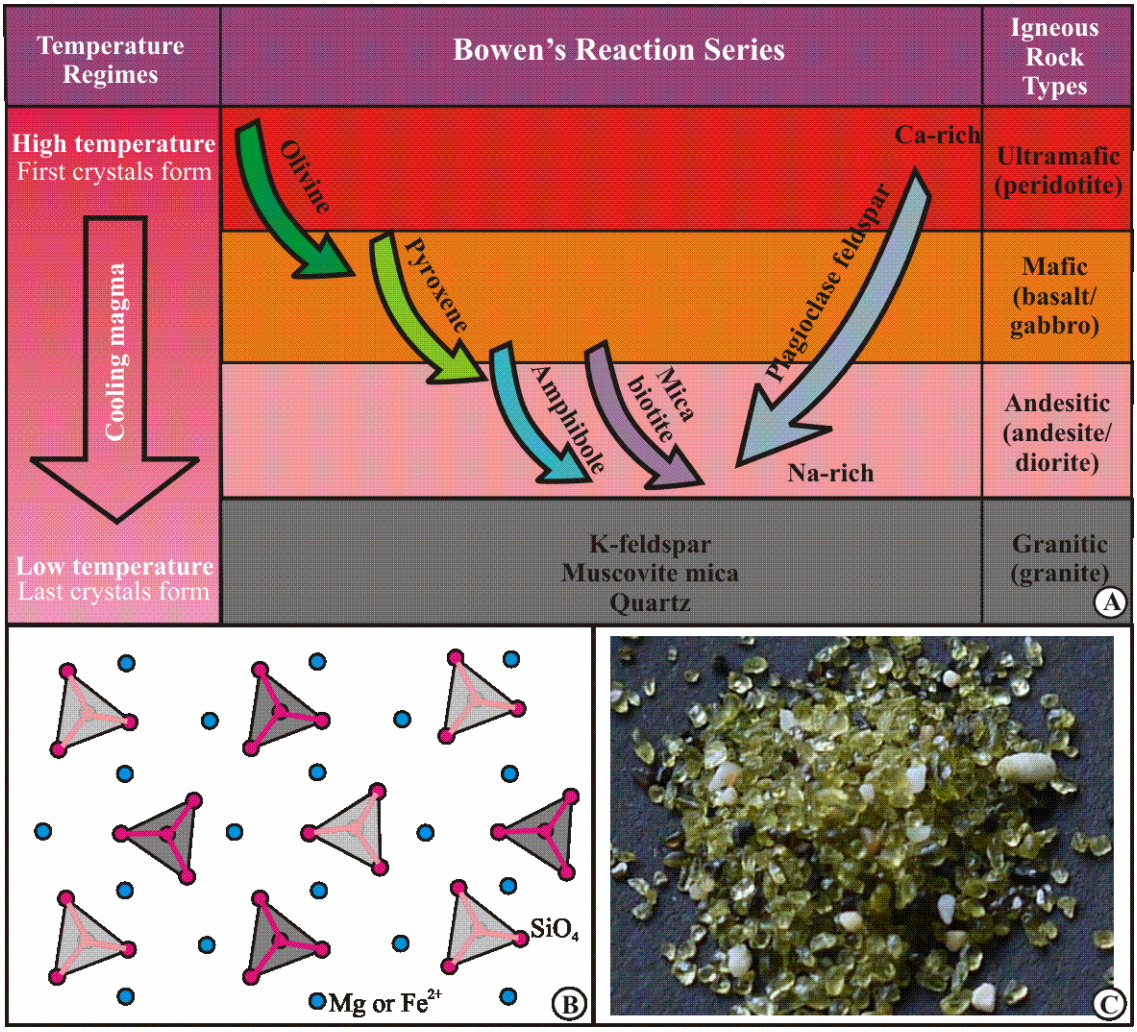

Figure 2.1. A) Bowen's reaction series of igneous mineral formation according to temperature regimes in cooling magma. The olivine series (broken arrows) is discontinuous, while plagioclase is continuous. Igneous rock types that each mineral phase is found in are listed at right. B) Atomic structure of olivine, with Mg or Fe^{2+} as blue dots interspersed between silica (SiO_4) tetrahedra. C) Olivine sand from Hawaii.

Growth of microbes on olivine

Even though olivine is an abundant mineral that is a rich source of Fe^{2+}, there are relatively few reports of microbes grown with olivine in the laboratory [*Santelli et al*, 2001; *Welch and Banfield*, 2002; *Shirokova et al*., 2010; *Garcia et al*., 2005; *Longazo et*

al., 2001, 2002; *Josef et al.*, 2007]. No studies to date have been published on the microbiology of olivine, or the microbial physiologies and phylotypes associated with olivine minerals in nature. Of the laboratory studies, only those by *Santelli et al*. and *Welch and Banfield* used olivine as the sole source of energy. Most of the studies were related to the effect of bacteria on the weathering of olivine. Interestingly, a few studies indicate that the presence of bacteria inhibits the dissolution of olivine by decreasing surface reactivity [*Santelli et al*, 2001; *Welch and Banfield*, 2002; *Josef et al.*, 2007]. Although, *Garcia et al*. [2005] found that this was not the case with *E. coli*, and *Longazo et al.* [2001, 2002] reported olivine bioweathering features when incubated with a basalt aquifer bacillus strain.

Organisms that were previously grown on olivine in the laboratory are *Acidothiobacillus ferrooxidans*, an unidentified bacillus from a Columbia River basalt aquifer, a consortium of environmental microbes, *E. coli*, and a Pseudomonad. *A. ferrooxidans* is a well-known acidophilic iron oxidizer while Pseudomonads are heterotrophic bacteria whose lineage includes some organisms that are capable of neutrophilic iron oxidation [*Straub et al*., 1996]. With the possible exception of the bacillus from a basalt aquifer, none of the organisms reportedly grown on olivine were isolated from olivine or olivine-bearing rocks.

Iron oxidizers

Iron oxidizers are common in most environments, and can live in anaerobic or aerobic conditions, acidic or neutral pH, freshwater or saline environments, and inhabit a wide

range of temperatures [*Konhauser et al*., 2011; *Emerson et al*., 2010]. Iron oxidizers can be autotrophs or heterotrophs, and some are even phototrophs [*Konhauser et al*., 2011]. Microorganisms that utilize the reduced form of iron (Fe^{2+}) are often represented in deep sea and terrestrial basalts, though a greater diversity of phyla have recently been recognized as having iron-oxidizing representatives [*Emerson et al*., 2002, 2010; *Smith et al*., 2011; *Konhauser et al.,* 2011; *Edwards et al.,* 2003a,b, 2004].

Iron oxidizers have been described from a variety of genera of *Bacteria* and *Archaea*. The most notable iron oxidizers are the archaeal genera *Ferroplasma* and *Ferroglobus,* and the bacterial genera *Gallionella, Leptothrix, Mariprofundus, Sphaerotilus, Sideroxydans, Acidothiobacillus, Leptospirillum, Acidovorax, Pseudomonas,* and *Marinobacter*. A variety of α-, γ- and ζ-*Proteobacteria* are newly-discovered iron oxidizers, yet they are not closely related to any previously known neutrophilic iron oxidizers [*Edwards et al*., 2004; *Emerson and Floyd*, 2005; *Duckworth et al*., 2009; *Wang et al*., 2009]. Even genera dominated by heterotrophic species such as *Pseudomonas* can contain strains that are obligate iron-oxidizing chemolithotrophs [*Bailey et al*., 2009]. Many iron oxidizers are autotrophic, but many are heterotrophic and use iron oxidation as an alternative source of energy [*Emerson et al*., 2002, 2010; *Konhauser et al.,* 2011; *Edwards et al.,* 2003a,b, 2004; *Straub et al*., 1996]. Iron oxidizers are often acidophilic or microaerophilic, since Fe^{2+} is more stable at low pH and the competition with abiotic iron oxidation lessens at low O_2 concentrations.

Neutrophilic iron oxidizers are common in basalts, but there are very limited reports of iron oxidizers that grow on olivine [*Stevens and McKinley,* 1995; *Emerson and Moyer,*

2002; *Edwards et al.*, 2003a,b; *Lehman et al*., 2004; *Emerson et al*., 2007; *Bailey et al*., 2009; *Santelli et al*, 2001; *Welch and Banfield*, 2002; *Shirokova et al*., 2010]. Discarded twisted stalks in iron mats on seamounts, hydrothermal deposits, or basalts produced by putative neutrophilic iron oxidizers are often the only evidence of the presence of iron oxidizers [*Edwards et al*., 2003a,b; *Emerson et al*., 2002, 2010; *Templeton*, 2011, *Orcutt et al*., 2010b].

Many recognizable iron oxidizers have adopted protective mechanisms to shield them from damaging free oxygen radicals and Fe^{3+} encrustation [*Emerson et al*., 2007]. Sheaths and stalks are common among neutrophilic iron oxidizers, yet no acidophilic iron oxidizer has been described yet with these features [*Edwards et al*., 2003a,b; *Hallbeck et al.*, 1993; *Emerson et al*., 2002; *Templeton*, 2011]. Sheaths have been described in freshwater *Leptothrix* sp., and stalks are produced by freshwater *Gallionella* and deep sea *Mariprofundus ferrooxydans* [*Emerson et al*., 2002; *Hallbeck and Pedersen*, 1995]. Sheaths and stalks are common in iron mats at deep sea hydrothermal vents, seamounts, and seafloor basalts [*Emerson et al*., 2002; *Orcutt et al*., 2010b; *Lysnes et al*., 2004], but *Mariprofundus ferrooxydans* is currently the only known iron oxidizer from the sea that produces extracellular stalks.

It is unclear what the primary purpose of stalk or sheath material is, but it is known that the material is periodically discarded [*Hallbeck et al*., 1993; *Hallbeck and Pedersen*, 1995; *Emerson et al*., 2007]. Stalks are believed to be made of an extensive network of polysaccharides [*Hallbeck and Pedersen*, 1995], which appear thermodynamically disadvantageous to produce. *Hallbeck and Pedersen* [1995] studied the effects of stalk

production in *Gallionella ferruginea* and found that stalks are not required for survival, but they do provide an advantage. A stalk-forming strain of *Gallionella* not only lived longer under oxygenic conditions, it also out-competed a non stalk-forming strain in a mixed culture. Using microscopy and spectroscopy to investigate stalk formation in *Mariprofundus ferrooxydans*, *Chan et al*. [2007] showed that while cells are growing in culture, they excrete iron and organic-rich fibrils which constitute stalk material at a rate of approximately 2 μm per hour. They also determined that stalk growth parallels iron and oxygen gradients.

Iron oxidation

Iron oxidation yields little energy for growth thermodynamically, hence chemolithoautotrophic iron oxidizers generally have a slow growth rate. Iron oxidation is favorable at both neutrophilic and acidic conditions, although more energy can be yielded at neutral pH. The redox potential ($E^{o'}$) of the Fe^{3+}/Fe^{2+} couple is pH-dependent and becomes more positive in acidic conditions, so iron oxidation is more exergonic at neutral pH than at acidic pH [*Thauer et al*., 1977; *Widdel et al*., 1993]. .

$$Fe^{2+} + \tfrac{1}{2}O_2 + 2H^+ \rightarrow Fe^{3+} + H_2O \qquad \textbf{\textit{Eq.1}}$$

at pH 2 the $E^{o'}$of Fe^{3+}/Fe^{2+} = + 0.77 V and $\Delta G^{o'}$= - 8.7 kJ per mol Fe

$$Fe^{2+} \text{ (as } FeCO_3) + \tfrac{1}{2}O_2 + 2H_2O \rightarrow Fe^{3+}(OH)_3 + H^+ \qquad \textbf{\textit{Eq.2}}$$

at pH 7 the $E^{o'}$of Fe^{3+}/Fe^{2+} = + 0.20 V and $\Delta G^{o'}$= - 63.7 kJ per mol Fe

Straub et al. [1996] reported that at pH 7 and in the presence of CO_2 and HCO_3^-, Fe^{2+} is primarily in the form of iron carbonate and the redox potential of the $Fe^{3+}(OH)_3 + HCO_3^-$/ $FeCO_3$ couple may be as low as +0.1V.

The complete oxidation of 1 mol of Fe^{2+} to $Fe^{3+}OOH$ or $Fe^{3+}(OH)_3$ at pH 7 yields 2 mol of H^+ [*Emerson and Moyer*, 1997]. Under acidic conditions, it is estimated that 120 moles of Fe^{2+} are needed to synthesize one mole of glucose [*Konhauser*, 2007], therefore large amounts of Fe^{2+} must be utilized for growth.

Oxidants other than gaseous O_2 are readily available in nature that can be used to oxidize iron. Nitrate is commonly used by some iron-oxidizing bacteria as an alternative electron acceptor in anaerobiosis [*Straub et al*., 1996; *Edwards et al*., 2003a]. Anaerobic nitrate-dependent iron oxidation relies on the nitrate reductase enzyme, which is downregulated in the presence of oxygen; hence it can only be used in anaerobic conditions. During nitrate reduction, nitrate (NO_3^-) is reduced to nitrite (NO_2^-), with an $E^{o'}$ value +0.43V [*Thauer et al.*, 1977; *Straub et al*., 1996]. Nitrite can be further reduced through nitrite reduction to NO ($E^{o'}$= +0.35V), NO reduced to N_2O ($E^{o'}$= +1.18V), and N_2O to N_2 gas ($E^{o'}$= +1.35V) [*Thauer et al.*, 1977; *Straub et al*., 1996]. Nitrate reduction all the way to N_2 gas is called denitrification, and is favorable in neutral to alkaline conditions. When the electron acceptor is nitrate, iron oxidation is endergonic at acidic pH (unfavorable) and exergonic at circumneutral pH (favorable).

$$2Fe^{2+} + NO_3^- + 2H^+ \rightarrow 2Fe^{3+} + NO_2^- + H_2O \qquad \textbf{\textit{Eq.3}}$$

at pH 2, $\Delta G^{o'}$= + 32.8 kJ per mol Fe

at pH 7, $\Delta G^{o'}$= - 22.2 kJ per mol Fe

Iron oxidation coupled with complete denitrification is also thermodynamically favorable at neutral pH [*Weber et al*., 2006].

Earth's crust

There are two general types of Earth's crust, oceanic and continental crust, which have very different properties. Oceanic crust, which comprises ~60% of the total crust, is mainly igneous rock derived from the upper mantle. These iron (Fe) and magnesium (Mg) rich young basalts are dense, yet their porosity allows for subsurface fluid flow [*Elderfield et al*., 1999; *Walker et al*., 2008; *Fisher et al*., 2003; *Hutnak et al*., 2006]. The ocean crust contains the largest continuous aquifer on Earth and seawater circulates within the ocean crust on the order of hundreds to thousands of years [*Elderfield et al*., 1999; *Walker et al*., 2008; *Fisher et al*., 2003; *Hutnak et al*., 2006; *Orcutt et al*., 2010a, 2011]. The ocean crust is layered with sediments, pillow basalt, intrusive basalt, and gabbro overlying peridotites. Continental crust contains the oldest rocks on Earth, is thicker than oceanic crust, and contains more silicon-rich rocks than ocean crust.

Flow cell crustal sampling

Sampling the subsurface biome comes with a unique set of challenges. Access to subsurface rock requires either costly drilling expeditions or exposure of subsurface rock in caves or mines. Contamination is an issue, and recent studies have shown that microbes in the host rock differ substantially from those free-living in the aquifer water [*e.g*., *Lehman*, 2007]. Microbes may also preferentially attach to specific minerals in the

host rocks, but current analytical techniques do not include investigating microbial ecology on the mineral level. A variety of devices have been developed to trap or enrich for microbes on rock surfaces incubated in subsurface boreholes and tunnels [*Moser et al.*, 2003; *Lehman*, 2007; *Ekendahl et al*., 1994; *Ekendahl and Pedersen*, 1994; *Pedersen et al*., 1996; *Dodds et al*., 1996; *Stevens et al*., 1993]. However, investigating the microbial preference for minerals *in situ* led to the development of flow-through cells that allow for microbial colonization of target mineral substrates [*Fisher et al*., 2005a; *Smith et al*., 2008; *Orcutt et al*. 2010a,b]. This approach combines monomineral substrates in isolated chambers with osmotically-driven pumps that draw formation water through the chambers. Once the chambers are recovered, minerals can be analyzed for degree of microbial colonization, mineral weathering, and phylogenetic and physiological diversity.

Life in Earth's crust

The subsurface biosphere harbors representatives from all domains of life, although *Bacteria* and *Archaea* appear to inhabit a wider range of endolithic environments than eukaryotes. Over ten years ago, it was estimated that up to sixty percent of *Bacteria* and *Archaea* live below the Earth's surface, as many as 6×10^{30} cells [*Whitman et al*., 1998]. The diversity of endolithic microbes and the limits of their subsurface habitats are still being explored, however it is becoming clear that subsurface microbes are surprisingly diverse and are found at great depths. Microorganisms in the deep subsurface have been discovered as far as 1.5 km deep in oceanic crust, and up to 4 km in terrestrial crust

[*Mason et al*., 2010; *Konhauser*, 2007]. Below these depths, the heat radiating from the Earth's mantle approaches the upper temperature limit of life, 120^{o} C [*Konhauser*, 2007].

Lava tubes as Mars-analogue environments

Lava tubes are tunnel-like basalt caves that are the remains of underground lava flows. They are often cold enough to house perennial ice, and the interface between ice and basalt in such oligotrophic, aphotic environments are excellent analogues for Mars subsurface habitats. Life below the surface of Mars would be sheltered from intense radiation and superoxides, protected from temperature extremes and dust storms, and have access to a warmer and possibly wetter environment more conducive to life [*Fogg*, 1996; *Abramov and Kring*, 2005; *Travis et al*., 2003; *Clifford et al*., 2010; *Fairén et al*., 2010; *Samarkin et al*., 2010; *Cushing et al*., 2007].

Over the past decade, lava tubes have been discovered on Mars near Arsia Mons, Olympus Mons, Pavonis Mons, east of Jovus Tholus, and Elysium Mons volcanoes [*Boston et al.*, 2011; *Cushing et al*., 2007]. Sheltered habitats such as these could allow for preservation of past or present microbial mats, mineral weathering features that are evidence of biological activity, unaltered sediments, trapped volatiles and organics, and allow the buildup of gases from rock formations below [*Boston et al.*, 2011].

Mars habitability

Surface temperatures on Mars fluctuate widely, but are largely below the freezing point of water. The thin atmosphere also leaves the surface exposed to harmful cosmic

and solar radiation. However, the Martian subsurface would be more hospitable to life since the temperatures are closer to freezing and rock and dust would shelter life against ionizing radiation. Subsurface temperatures could have even been above freezing in the recent geological past because of residual geothermal heat [*Fogg*, 1996; *Abramov and Kring*, 2005]. Liquid water could have existed on Mars over much of the planet's history, and may still exist in the subsurface at the rock-ice interface, in rocks and soils, after impact events, and in brines [*Travis et al*., 2003; *Clifford et al*., 2010; *Fairén et al*., 2010; *Samarkin et al*., 2010]. Chemolithotrophic life could exist in the Martian subsurface using reduced igneous minerals (which are abundant on the volcanic world) and electron acceptors such as superoxides in surface dust or atmospheric O_2.

Biological weathering patterns in basalt

Alteration textures due to the bioweathering of basalt has been described in many forms, such as etching, pitting, and microchannel or tubular texture formation [*Fisk et al*., 1998, 2003, 2006; *Thorseth et al*., 2001, 2003; *Torsvik et al*., 1998; *Welch and Banfield* 2002; *Santelli et al*., 2001; *Furnes et al*., 2001, 2004; *Staudigel et al*., 2008; *Storrie-Lombardi and Fisk*, 2004; *Banerjee et al*., 2003, 2006, 2011; *Garcia et al*., 2005; *Longazo et al*., 2001, 2002; *Josef et al*., 2007, *Izawa et al*, 2010; *Kruber et al*., 2008, and others]. Tubular and granular formations in basalt have never been unequivocally proven to be a direct result of microbial activity since no microbe is known that can produce these structures. The oldest known microchannel (including tubular and granular) formations are from the Barberton Greenstone Belt in South Africa [*Banerjee et al*.,

2003, 2006]. The Barberton Greenstone Belt contains pillow lavas from the Archaean, and it is estimated that microbes colonized the volcanic rocks between 3.4 – 3.5 billion years ago [*Banerjee et al*., 2003, 2006]. One study found the presence of organic carbon and partially oxidized iron within the microchannel-like alteration textures, indicating they are of microbial origin [*Benzerara et al*., 2007].

There are multiple types of alteration textures, varying in size, shape, and form [*Banerjee et al*., 2003, 2006, *Fisk et al*., 1998, 2003, 2006; *Furnes et al*., 2004; *Staudigel et al*., 2008; *Kruber et al., 2008,* and others]. *Staudigel et al.* [2008] proposed mechanisms for formation of both granular and tubular alteration textures; however without a biological organism to produce these features, all theories remain unproven. Multiple textural types may indicate that a multitude of microbial phylotypes (or physiotypes) are capable of producing alteration textures, although it is unclear whether the microorganisms are utilizing oxidation or reduction of elemental components of basalt glass, or simply using a physical boring technique. Microchannels have been described in the Fe^{2+}-containing minerals olivine and pyroxene [*Fisk et al.*, 1998, 2003, 2006], which indicates some microchannels may be formed by iron-oxidizing bacteria. Bioweathering textures are also found in a wide variety of basaltic rocks including oceanic crust, terrestrial flood basalts, and possibly the Nahkla meteorite from Mars [*Fisk et al.*, 1998, 2003, 2006; *Fisk and Giovannoni* 1999]. With the exception of the Martian meteorite, the interiors of microchannels have tested positive for DNA, phosphorous, potassium, aromatic amino acids, and organic carbon, providing supporting evidence that microchannels are formed by microbes [*Fisk et al*., 2006; *Benzerara et al*., 2007].

If putative microbial alteration textures in igneous minerals and glasses can be proven to be the direct result of microbial activity in volcanic rocks on Earth, they would represent a new astrobiological and micropaleontological biosignature that could be used in the search for life on Mars or early life on Earth. Alteration textures found in the Nahkla meteorite remain inconclusive evidence of life because they are solely based on visual appearance, and not in conjunction with other biosignatures such as DNA or amino acids. Also, the microtextures seen in Nahkla appear slightly different than the microchannels found in basalt glass. The channels perpendicular to the alteration front are all straight and parallel to each other, which may likely be a product of the natural abiotic weathering of olivine [*Welch and Banfiled,* 2002].

CHAPTER 3

In situ enrichment of ocean crust microbes on igneous minerals and glasses using an osmotic flow-through device

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Smith, A., R. Popa, M. Fisk, M. Nielsen, C. G. Wheat, H. W. Jannasch, A. T. Fisher, K. Becker, S. M. Sievert, and G. Flores (2011), In situ enrichment of ocean crust microbes on igneous minerals and glasses using an osmotic flow-through device, *Geochem. Geophys. Geosyst.*, 12, Q06007, doi:10.1029/2010GC003424.

Abstract

The Integrated Ocean Drilling Program (IODP) Hole 1301A on the eastern flank of Juan de Fuca Ridge was used in the first long-term deployment of microbial enrichment flow cells using osmotically-driven pumps in a subseafloor borehole. Three novel osmotically-driven colonization systems with unidirectional flow were deployed in the borehole and incubated for four years to determine the microbial colonization preferences for twelve minerals and glasses present in igneous rocks. Following recovery of the colonization systems, we measured cell density on the minerals and glasses by fluorescent staining and direct counting and found some significant differences between mineral samples. We also determined the abundance of mesophilic and thermophilic culturable organotrophs grown on marine R2A medium and identified isolates by partial 16S or 18S rDNA sequencing. We found that nine distinct phylotypes of culturable mesophilic oligotrophs were present on the minerals and glasses and that eight of the nine can reduce nitrate and oxidize iron. Fe(II)-rich olivine minerals had the highest density of total countable cells and culturable organotrophic mesophiles, as well as the only culturable organotrophic thermophiles. These results suggest that olivine (a common igneous mineral) in seawater-recharged ocean crust is capable of supporting microbial communities, that iron oxidation and nitrate reduction may be important physiological characteristics of ocean crust microbes, and that heterogeneously distributed minerals in marine igneous rocks likely influences the distribution of microbial communities in the ocean crust.

Introduction

Much of the earth's microbial biomass exists in the subsurface, and a sizable fraction of this is in ocean sediments and igneous rocks [*Whitman et al*., 1998]. If microbial life in the igneous ocean crust extends to the depth of the ~120°C isotherm (commonly thought to be the upper temperature limit of life) then the volume of rock available for microbial colonization is about the same as the volume of the oceans [*Heberling et al*., 2010]. Most of this volume is difficult to sample but microorganisms have been identified from igneous layers of the ocean crust [*e.g. Mason et al*., 2010]. Additional understanding of the deep igneous biome comes from microorganisms extracted from subsurface fluids [*e.g. Cowen et al*., 2003], although unattached microorganisms in aquifer fluids are not necessarily representative of the microorganisms attached to the host rocks [*Lehman*, 2007]. Microbes may also preferentially attach to specific minerals in the host rocks, but because oceanic igneous rocks are typically mineralogically heterogeneous on the 0.01 to 10 millimeter scale and cell abundances are low, current analytical techniques are inadequate for determining what microorganisms are associated with specific minerals.

A new approach for determining microbial preference for igneous minerals uses *in situ* colonization of target mineral substrates housed in flow-through cells [*Fisher et al*., 2005a; *Smith et al*., 2008; *Orcutt et al*. 2010a,b]. This approach combines monomineral substrates in isolated chambers with osmotically-driven pumps that draw formation water through the chambers. Once the chambers are recovered, each mineral type can be processed to determine the degree of microbial colonization, the phylogeny of

community members, community complexity and microbial diversity, physiology using culture based studies, and mineral weathering. Here we report initial findings from a four year flow cell incubation in the deep ocean subsurface of the eastern flank of the Juan de Fuca Ridge (JFR). This is one of two experiments of this type [*Orcutt et al.*, 2010a,b; *Smith et al*., 2008], although it is the first to incubate a wide diversity of igneous minerals using fluid drawn across the samples with an osmotic pump.

Subsurface microorganisms are diverse and include all domains of life (*Bacteria*, *Archaea*, and *Eukarya*) and appear to colonize ocean crust basalt via subseafloor fluid transport [*Huber et al*., 2006; *Santelli et al*., 2008; *Mason et al*., 2010]. Microbes living in seafloor and subseafloor volcanic environments include psychrophiles, mesophiles, and thermophiles; aerobes and anaerobes; organotrophs and chemolithotrophs; and respiratory and fermentative autotrophs and heterotrophs. Microbial physiologies that are believed to be linked to geochemical cycles in subseafloor habitats include Mn(II) and Fe(II) oxidation [*Templeton et al*., 2005a, b; *Edwards et al*., 2003a, b; *Lysnes et al*., 2004], Fe(III) reduction [*Kashefi et al*., 2002; *Kashefi and Lovley*, 2003; *Ver Eecke et al*., 2009], methanotrophy [*Beal et al*., 2009], NH_4^+ oxidation [*Hatzenpichler et al*., 2008], NO_3^- reduction [*Cowen et al*., 2003, *Wheat et al*., 2010], heterotrophy [*Cowen et al*., 2003], hydrocarbon degradation [*Mason et al.*, 2010, *Kniemeyer et al*., 2007], sulfur oxidation [*Rathsack et al*., 2009], and sulfur and sulfate reduction [*Zhou et al*., 2009; *Nakagawa et al*., 2006; *Kniemeyer et al*., 2007]. Yet with few exceptions [*Orcutt et al*., 2010a,b], earlier analyses focused on microbes from whole basalt and had a limited capacity to describe the spatial distribution of microbial populations. In addition,

culturing and DNA-based studies may be inadequate to fully evaluate the role of subseafloor microbes in geochemical cycling and mineral weathering. To better understand the subseafloor distribution of microbial communities, new methods are required that combine monomineralic substrate incubations in subsurface water with cell density measurements, culturing, and DNA analyses. These methods will also help assess the impact of subseafloor communities on geochemical cycling of elements and the weathering of basalt [*Stevens*, 1997; *Thorseth et al*., 2001; *Daughney et al*., 2004; *Amend and Teske*, 2005; *Santelli et al*., 2008].

The microbiology of JFR is known from studies of surface and subsurface basalts, hydrothermal deposits, and thermal fluids. Fluids emanating from the 64°C IODP Hole 1026B contained nitrate reducers, sulfate reducers, and fermentative heterotrophs originating from the subsurface [*Cowen et al*., 2003]. Black rust formed by venting fluids at Hole 1026B contained similar organisms found in the subsurface fluid, and the community was dominated by fermenters, sulfate reducers, and methanogens [*Nakagawa et al*., 2006]. Also, electron microscopic examination of rocks collected from Hole 1026B revealed microorganisms associated with secondary minerals [*Fisk et al.,* 2000]. Samples collected from nearby 20°C Baby Bare springs were diverse but interestingly did not include a dominant organism from Site 1026B [*Huber et al*., 2006]. Mineral and basalt chips incubated in passive flow in Hole 1301A revealed DNA sequences largely from *Firmicutes,* yet SEM images indicate low-temperature iron oxidizers may have been present when seawater influx and cooler temperatures prevailed [*Orcutt et al*., 2010b]. Analysis of natural samples of basalt, metalliferous sediment, and sulfides that were

exposed to ~3°C bottom water revealed the presence of chemoautotrophic iron-oxidizing bacteria [*Edwards et al*., 2003a, b], as well as functional genes for carbon fixation, nitrogen fixation, methane oxidation, and methanogenesis [*Mason et al*., 2009]. Additional microbial diversity has been revealed from hydrothermal chimneys [*Schrenk et al*., 2003; *Ver Eecke et al*., 2009; and others]. Of these studies, those by *Cowen et al*. [2003], *Nakagawa et al*. [2006], *Orcutt et al.* [2010b], and *Huber et al*. [2006] provide insight into the microbiology of the subsurface igneous ocean crust. We hypothesized in crafting the present study that minerals with more available redox energy at the mineral-liquid interface will have a greater abundance of microbial cells attached to the mineral surface than the minerals with less available energy.

Materials and methods

Study site description. Juan de Fuca Ridge (JFR) is a zone of volcanic and hydrothermal activity on the Eastern Pacific rim [*Fisher et al*., 2005a]. On the eastern flank of JFR, the ocean floor consists of sediments overlaying igneous ocean crust [*Underwood et al*., 2005]. During IODP Expedition 301 in 2004, several ridge-flank boreholes were instrumented including Hole 1301A [*Fisher et al*., 2005a] (Figure 3.1A). Hole 1301A is situated at 47° 45.210' N, 127° 45.833' W where the water depth 2667 m and the upper volcanic crust is 3.5 m.y.old. The hole has total depth of 369.7 meters below the seafloor (mbsf) with the upper 262.2 m composed of marine sediment and the lower 107.5 m composed of basalt (Figure 3.1B). This basalt is typical of ocean crust Layer 2A (highly porous and permeable pillow lavas and sheet flows containing plagioclase feldspar,

pyroxene, and olivine) [*Fisher et al*., 2005a; *Becker et al*., 2008]. Upper basement rocks around Hole 1301A are characterized by elevated temperatures (~60 °C at the sediment-basalt interface) low oxygen, neutral to slightly alkaline *pH*, and an influx of nitrate-bearing (>10 μM) seawater [*Wheat el al*., 2010]. Although we did not test for total dissolved organic carbon (DOC) in 1301A fluids, nearby Baby Bare Spring and IODP Hole 1026B are reported to have low total DOC (10-15 μM) compared to bottom seawater (38 μM) [*Walker et al.*, 2008; *McCarthy et al.*, 2011, *Lang et al*., 2006]. Hole 1301A was fitted with a Circulation Obviation Retrofit Kit (CORK) [*Fisher et al*., 2005b], which was designed to seal the borehole from the ocean and to suspend instruments in the hole (Figure 3.1C). This provided a unique opportunity to study the differential microbial colonization of igneous phases *in situ*.

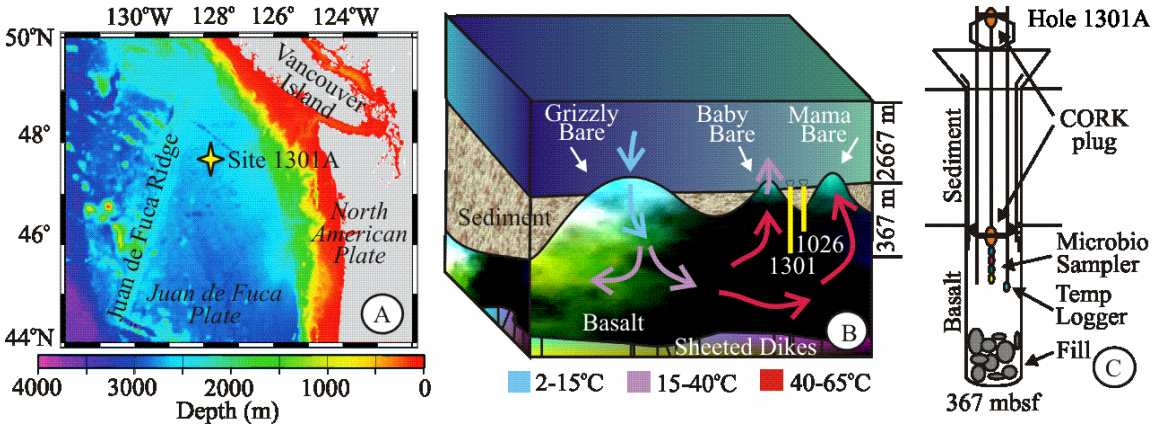

Figure 3.1. A) Location of IODP Site 1301A on the eastern flank of Juan de Fuca Ridge (modified from [*Fisher et al*., 2005b]). B) Stylized cartoon of fluid flow in the basalt basement near Hole 1301A indicated by blue (cool seawater) to red (hydrothermal water). Cold bottom seawater is predicted to enter the ocean crust at the Grizzly Bare outcrop, 52 km to the south, warms by lithospheric heating as it flows north-northeast toward Baby Bare and Mama Bare, where at least some of the water exits as springs from these outcrops [*Wheat el al*., 2000, 2002; *Fisher et al*., 2003]. This cycle is predicted to take up to hundreds of years to complete [*Elderfield et al*., 1999; *Walker et al*., 2008; *Fisher et al*., 2003; *Hutnak et al*., 2006]. C) Simplified diagram of Hole 1301A (modified from [*Fisher et al*., 2005b]) depicting the CORK and instrument string layout. Microbial flow cells were incubated in the basalt basement (Microbio Sampler) near a temperature logger.

Microbial flow cell design and implementation. Igneous specimens were acquired from various sources (Ward's Geology, Unimin Co., seafloor basalt glass). Because the osmotic system pumps low volumes of fluid (on the order of 30 mL per year), we minimized the amount of free volume and increased mineral surface area by using minerals and glasses that were crushed and sieved to obtain uniform grain sizes between 0.6 mm and 2.0 mm. Each flow cell held four mineral or glass specimens that were exposed to borehole water in sequence (Figure 3.2; Table 3.1) [*Fisher et al*., 2005; *Smith et al*., 2008]. The flow-through colonization systems described by *Orcutt et al*. [2010a] are similar in design and function to those used in this study [*Smith et al*., 2008]. The

flow cells are 67 mm in diameter and 70 mm tall PVC cylinders, each with four incubation chambers (13 mm in diameter and 42 mm deep cylindrical spaces milled into the PVC) lined with Teflon (Figure 3.2). We chose a closed system to avoid contamination of the minerals during deployment and recovery and to allow us to compare experimental samples to sterile controls. This closed system required a controlled water flow that was achievable through the addition of an osmotic pump to each flow cell pair. The osmotic pump design has the advantage of providing a low-rate continuous flow for several years [*Jannasch et al*., 2004]. "In series" and "in parallel" flow designs were considered, and we chose "in series" to eliminate the stagnation of fluid flow through the chambers with the lowest permeability (*i.e*., fluid channeling), which would happen if the chambers were arranged in parallel. Unequal permeability could occur by packing of minerals with different shapes or grain sizes and clogging of pore spaces with biological material or secondary minerals. Since our goal was to investigate microbial enrichment of a variety of igneous phases, using one osmotically-driven pump for each mineral sample and its control was unworkable in the space available in the borehole. The "in series" design also has the benefit of ensuring that all minerals in the "*Control*" and "*Experimental*" cells experience the same volume of water.

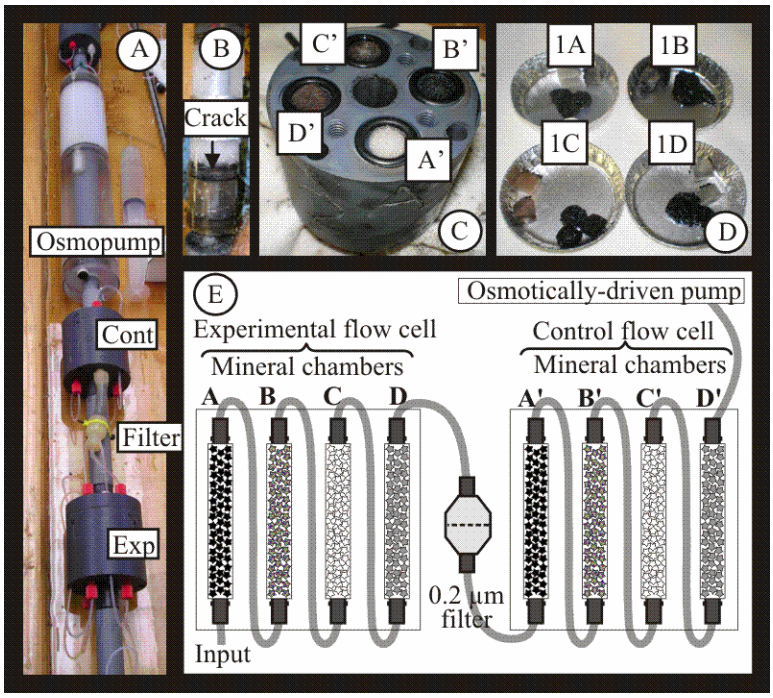

Figure 3.2. A) Hole 1301A string organization of one flow cell pair with an osmotically-driven pump shown prior to deployment (modified from [*Fisher et al*., 2005a]). B) One of the cracked osmotically-driven pumps, with NaCl solution remaining. C) Flow cell #2 opened to show the four chamber outlets (top side) with black o-rings and retaining sponges. Borehole water entered through the bottom of chamber A, exited through the top of chamber A (visible with white sponge), then entered the other three chambers in succession from bottom to top, eventually exiting the flow cell out of the top of chamber D. Sponge darkening in chambers B, C, and D is likely due to iron sulfide precipitates from H_2S reacting with the iron from olivines. Chamber A contained an iron-poor mineral. D) Mineral sands and retaining sponges recovered from flow cell #1. Discoloration of the sponges is visible. E) Diagram depicting one pair of flow cells (one "*Experimental*" and one "*Control*"). Each flow cell chamber contained one mineral (A, B, C or D). A', B', C' and D' minerals from the *"Control"* flow cells were the same as A, B, C and D. The minerals contained in each chamber are listed in Table 3.1.

All minerals and glasses were autoclaved before being placed in the chambers. Pairs of flow cells (1-2, 3-4, and 5-6) were designated "*Experimental*" (flow cells 1, 3, and 5) and *"Control"* (flow cells 2, 4, and 6). The "*Experimental*" and "*Control"* of each pair had the same sequence of minerals. The "*Experimental*" flow cells were exposed to

ambient borehole water while the water entering the *"Controls"* was sterilized using 0.2 micron filters. Each "*Experimental*/*Control*" flow cell pair was connected to an osmotically-driven pump [*Jannasch et al*., 2004]. The flow cell pairs were spaced ~3 meters apart between 275 to 287 mbsf and were placed in Hole 1301A on July 20, 2004, inside slotted steel casing near the base of the borehole observatory [*Fisher et al*., 2005b].

The flow cells were opened and the mineral chambers were emptied immediately upon being recovered and brought aboard R/V Atlantis in August 2008. Aseptic techniques were used in all procedures to avoid contamination from airborne or shipboard microbes. Minerals were separated into aliquots; some were used to inoculate media aboard ship, and the remainder stored at 4^{o}C and -40^{o}C. Mineral suspensions used to inoculate iron oxidizer/iron reducer media on board R/V Atlantis were stored at 4^{o}C and used for organotroph enrichments and cell counts upon return to the laboratory (~1 week later).

Table 3.1. Mineral and glass placement in the flow cells incubated in Hole 1301A. Borehole water was pumped through the flow cell mineral chambers in sequence from A through D, and then A' through D' as shown in Figure 3.2 for each flow cell pair. Mineral generic chemical formulas are given. Glasses do not have fixed formulas. Forsterite notation is given for olivines.

	Mineral Chamber			
Flow Cell Number	**A and A'**	**B and B'**	**C and C'**	**D and D'**
1 and 2	forsterite (Fo_{100}) Mg_2SiO_4	olivine (Fo_{90}) $Mg_{1.8}Fe_{0.2}SiO_4$	fayalite (Fo_0) Fe_2SiO_4	hornblende $Ca_2(Mg,Fe)_4Al(Si_7Al)O_{22}(OH)_2$
3 and 4	basalt glass	obsidian	augite $(Mg,Fe)CaSiO_6$	diopside $MgCaSiO_6$
5 and 6	anorthite $CaAl_2Si_2O_8$	bytownite $Na_{0.2}Ca_{0.8}Al_{1.8}Si_{2.2}O_8$	orthoclase $KAlSi_3O_8$	apatite $Ca_5(PO_4)_3OH$

Enrichments for thermophilic iron oxidizers and iron reducers. The enrichment media described below were inoculated aboard ship within four hours of recovery. Enrichments for iron oxidizers and iron reducers were prepared in Hungate tubes using 10 ml of *pH* 8 Artificial Sea Water (ASW) medium modified from *Emerson et al.* [2007]. The ASW medium contained: 470 mM NaCl; 20 mM Na_2SO_4; 1.5 mM KCl; 1 mM $NaHCO_3$; 1 mM $MgCl_2$; 0.5 mM NH_4Cl; 0.37 mM K_2HPO_4; 20 mL/L Wolfe trace elements mix; and 1 ml/L Wolfe vitamins mix. The Wolfe trace elements mixture contained: 12 mM Mg^{2+}; 12 mM SO_4^{2-}; 0.25 mM Mn^{2+}; 0.37 mM Fe^{3+}; 0.42 mM Co^{2+}; 0.68 mM Ca^{2+}; 0.37 mM Zn^{2+}; 0.118 mM Cu^{2+}; 21 μM Al^{3+}; 0.021 mM K^{+}; 0.16 mM BO_3^{3-}; and 41μM Mo^{6+}. The Wolfe vitamins mixture contained 5 μg/mL p-aminobenzoic acid; 5 μg/mL biotin; 5 μg/mL cyanocobalamin; 5 μg/mL folic acid; 100 μg/mL i-inositol; 100 μg/mL nicotinic acid; 100 μg/mL pyridoxine; 100 μg/mL panthotenic acid; 100 μg/mL riboflavin and 1 μg/mL thiamine. Iron oxidizer enrichments contained 1 gram of Fo_{90} olivine as the reductant and 1% O_2 as the oxidant. Iron reducer enrichments were based on media described previously [*Kashefi et al.,* 2002] and contained 10 mM ferric chloride as the oxidant, with 5 mM acetate and 5 mM lactate as reductants. The iron reducer enrichments were incubated under a N_2 atmosphere containing an initial concentration of ~1.6% O_2. All enrichments were inoculated with 100 μL of "*Experimental*" mineral suspensions from tubes containing 50 mg mineral sand and 250 μL 0.1 micron-filtered sterile seawater, then immediately placed into a 55°C incubator. After transfer to the laboratory, all iron-dissimilating microbial enrichments were incubated at 55°C for up to 6 weeks. Growth was monitored over the incubation period using DAPI staining of

microbes in suspension after vigorous vortexing for 15 minutes and epifluorescent microscopic counting [*Bennett et al.*, 2006].

Isolation and identification of aerobic oligotrophs. Approximately 1 gram of each mineral specimen was aseptically recovered from each chamber of the flow cells aboard ship and stored at 4^{o}C until plated in the laboratory (approximately 1 week later). Measured amounts of minerals and filter-sterilized seawater were mixed, vortexed vigorously for 15 minutes, then immediately used to produce serial dilutions for culturing oligotrophs on plates and total direct counting. The immediate transfer ensured that microbes immobilized on very fine suspended mineral particles were also included in the serial dilution. Serial dilutions were plated in triplicate on Marine R2A (MR2A) media (Difco R2A agar Catalog #218263 plus 27.5 g/L NaCl), a low-organic solid media for isolating oligotrophs. MR2A plates were incubated at 55°C and 25°C for up to one week. The Colony Forming Units (CFUs) were counted and averaged across triplicates. Dilutions containing between 30-500 colonies were used for calculating CFU/mL and then CFU/g of mineral.

Eight colonies with dissimilar morphology were selected per mineral for DNA sequencing. Isolates were numbered named with numbers corresponding to the flow cell number and letters corresponding to the mineral chamber (Table 3.1). Plates from basalt glass and apatite were subsequently lost due to fungal contamination during later transfers and were not included in DNA analysis. Libraries of isolated colonies were saved (-80°C in 50 % glycerol). For phylotyping, the isolated colonies were scraped from plates and the genomic DNA (gDNA) was extracted in 99°C TE buffer for ten

minutes [*Cook and Meyers*, 2003; *Mazza et al*., 2003]. The concentration of gDNA was quantified with a NanoDrop 1000 spectrophotometer. Supernatants containing DNA were used as a DNA template in a Polymerase Chain Reaction (PCR) targeting the 16S rRNA gene using either the bacterial 8F (5'AGAGTTTGATCCTGGCTCAG) or archaeal 4F (5'TCCGGTTGATCCTGGCRG) primers; and 1492R (5'GGTTACCTTGTTACGACTT) [*Baker et al*., 2003]. Only a few microbial isolates (<5%) having very low gDNA yields were not successfully amplified with this combination of primers. All PCR amplifications used a PCR kit (Fermentas) and a GeneAmp 2400 thermocycler (Perkin Elmer), and the following conditions (95°C for 3 minutes, 40 cycles of: 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, then a final 72°C for 5 minutes). The PCR products were verified by agarose gel electrophoresis, cleaned by using a PCR cleanup kit (Qiagen) and sequenced using an ABI 3130xl sequencer at the Oregon Health Sciences University DNA Core Facility.

Sequence files were trimmed to between 300 and 350 nucleotides depending on sequence quality. Trimmed sequences were aligned and clustered into operational taxonomic units (OTU) at 97% sequence similarity using tools available through the Ribosomal Database Project (RDP) [*Cole et al*., 2009]. Representative sequences of each OTU were chosen to receive nearly full-length 16S rRNA gene sequencing using the primers 5F, 515F, and 1492R. Sequence fragments were manually aligned in MEGA4 and all representative sequences were aligned and trimmed to 1269 nucleotides. Representative sequences were then imported into ARB [*Ludwig et al*., 2004], aligned according to secondary structure constraints, and added to the Silva-96 reference tree

[*Pruesse et al*., 2007]. 16S rRNA gene sequences of cultured organisms closely related to our isolates and those of similar organisms from ocean crust habitats were selected and all sequences were imported into and aligned with MEGA4 [*Tamura et al*., 2007]. Phylogenetic analysis was conducted in both ARB and MEGA4 on only unambiguous nucleotide positions (636 bp). A phylogenetic tree was generated using the neighbor-joining method [*Saitou and Nei*, 1987] with 500 bootstrap replications in MEGA4. Tree topologies from MEGA4 were identical with those from ARB.

Fixing cells from mineral surfaces. Mineral samples were fixed on board R/V Atlantis following procedures described previously [*Bennett et al*., 2006]. Immediately after opening the flow cells, approximately 250 mg of mineral samples were placed in sterile 1.5 mL tubes and rinsed gently with 1x Phosphate-Buffered Saline (PBS) by inverting very gently and slowly. The supernatants were removed with a pipette and saved in 1.5 mL microcentrifuge tubes. The remaining minerals were prepared for staining by mixing 1 volume of the sample with 3 volumes ice-cold 4% paraformaldehyde (PFA). Samples were allowed to fix at 4^{o}C for 12 hours. After fixing, the supernatant was removed and saved again, then 4 volumes 1x PBS was added down the inside of the tube to leave crystals undisturbed. The fluid in the tubes was gently mixed by pipetting up and down. This washing step was repeated three times. Samples were resuspend in 1 volume 1x PBS and an additional 1 volume of ice-cold 96% (v/v) ethanol was added to the tubes. All samples were placed at -20^{o}C until stained in the laboratory. Cells from 10 μL aliquots of removed fixative, washes, and the fixed mineral samples were mixed with 90 μL of a 5 μg/mL DAPI (4', 6-diamidino-2-phenylindole) solution in PBS and incubated

for 5 min [*Bennett et al*., 2006]. Cells were visualized with an epifluorescent microscope (Carl Zeiss, Axioscope).

DAPI staining of cells from mineral specimens. Methods of counting cells on minerals from environmental samples were followed as described previously [*Bennett et al.*, 2006]. To obtain total cell counts, 10 μL aliquots of the vortexed serial dilutions were suspended in 90 μL of filter-sterilized 1x (PBS) buffer. Aliquots of this suspension (10 μL) were mixed with 90 μL of DAPI-PFA staining solution (5 μg/mL of 4', 6-diamidino-2-phenylindole solution in PBS with 4 % paraformaldehyde) and incubated for 5 min [*Bennett et al*., 2006]. One ml of PBS was added to each cell suspension and the mixtures were transferred to black polycarbonate 0.2 μm filters (Poretics), and washed with 2 mL of PBS buffer. Cell numbers were counted using an epifluorescent microscope (Carl Zeiss, Axioscope) then expressed in cells g-mineral^{-1}.

Petrographic thin sections. Samples were placed in sterile metal weigh dishes then 100 μL of DAPI solution (see ***DAPI staining of cells from mineral specimens***) was added for each 1 mL of liquid that remained with the mineral samples after transfer from the flow cells. Samples were kept in the dark for all subsequent steps to avoid photo-degradation of DAPI stain. Samples were incubated at room temperature for 10 minutes, gently swirling a few times to mix. One volume of 95% ethanol was added to fix cells to minerals and incubated for 5 minutes. One volume of ddH_20 was added and swirled gently to mix, and then the liquid was decanted. Samples were rinsed with 5 volumes ddH_2O twice more and decanted again. A sterile pipette was used to remove any remaining excess liquid. Samples were allowed to dry in a warm, dark, oven overnight

covered with aluminum foil. Samples were kept in foil-wrapped 1.5 mL tubes until returned to the lab. Petrographic thin sections of mineral sands were prepared without heating at OSU using Epo-Tek 301 low-fluorescence resin. Thin sections were viewed under light and fluorescent microscopy to visualize cells and search for mineral weathering patterns.

Physiological screening of organotrophs for iron oxidation and nitrate reduction. All phylotypes were evaluated for the ability to reduce nitrate and nitrite and to oxidize Fe(II). For the nitrate reduction test, we prepared a marine 10mM nitrate broth (DIFCO Catalog #226810 plus 470 mM NaCl) in culture tubes containing an inverted Durham tube to capture any gas produced from denitrification. After 5 days incubation at 30^{o}C, cultures were analyzed for evidence of denitrification, and nitrate and nitrite reduction as described previously [*Leboffe and Pierce*, 2005]. Strains that produced gas while growing in nitrate broth were also tested for carbohydrate fermentation using Triple Sugar Iron Agar [*Leboffe and Pierce*, 2005]. Iron oxidation was verified using gradient tubes with a 30 mM $FeCO_3$ 2% agar plug at the bottom of the tube. The 0.5% soft agar overlay contained the marine mineral medium described above supplemented with 30 mM NO_3^- as an alternate electron acceptor and 2 mg/L resazurin redox indicator. Tubes were incubated at 25^{o}C for four days. Iron oxidation was confirmed by the presence of a discrete growth band in the soft agar gel that was not present in the negative control. The increased abundance of microbes in the bands was verified by microscopy. Strains that did not produce a growth band with cells or remained identical to the uninoculated tubes were assumed to be negative for iron oxidation capabilities.

Growth of selected strains on olivine and nitrate. Four strains related to known iron oxidizers were chosen to determine their ability to grow on olivine in a mineral medium containing nitrate as the oxidant. 1 gram of washed Fo_{90} olivine sand was placed in Hungate tubes containing 6 ml of *pH* 8 ASW medium. ASW medium content was as described in ***Enrichments for thermophilic iron oxidizers and iron reducers***, except for the following additions: 10 mM total $NaHCO_3$ and PO_4^{3-}, 10 mM NO_3^-, 1 mM acetate, and 3 mM L-cysteine hydrochloride as a reducing agent. Tubes were sealed and crimped with 1 cm butyl stoppers and bubbled with N_2 gas containing 1.6% O_2. After autoclaving, tubes were inoculated with either *Nitratitreductor* strain 3d.4, *Halomonas* strain 1c.1, *Marinobacter* strain 1c.10, *Pseudomonas* strain 1b.2, or freshwater aquarium soil enrichments. Prior to inoculation, all organisms were grown in marine R2B for 5 days at 25^{o}C then centrifuged at 1250 rpm for 10 minutes. Media was removed with a pipette and cell pellets were washed with 1 mL ASW medium. 0.5 mL of this suspension was injected into the culture tubes using a 1 mL syringe. Tubes were incubated at 30^{o}C for up to one month. Cell counts were taken on days 5 and 22 using a microscopic counting chamber.

Marinobacter *strain 3d.6 O_2-respiration and Fe(III) reduction on olivines.*

Marinobacter strain 3d.6 cells were grown for 5 days at 25^{o}C in marine R2B medium supplemented with 1 mM $FeSO_4$. Cells were pelleted by centrifugation and washed in ASW. Cell suspensions were injected into sterilized 140 mL sealed and crimped culture jars containing 1 gram washed Fo_{90} or Fo_0 olivine sand and *pH* 7.3 ASW medium under 21% O_2 atmosphere. Oxygen evolution was monitored weekly for 8 weeks, then again at

22 weeks. O_2 concentration in the head space was measured by gas chromatography using an SRI 310C instrument, a Molecular sieve column, and a TCD detector. The gas pressure was measured with an Omega pressure meter (Omega Engineering, Inc. Ct).

For Fe(III) reduction testing, media were prepared as above except the jars were flushed with N_2 gas containing 1.6% O_2. A mix of LASP (Na lactate, Na pyruvate, Na acetate, and Na succinate) (5 mM final concentration) were added as potential reductants to reduce Fe(III) on the surface of olivine crystals. Jars were incubated at 30^{o}C for 5 weeks, and each medium was analyzed by high-performance liquid chromatography (HPLC) weekly for changes in LASP. HPLC was performed with a DYNAMAX SD-300 instrument with ESA UV detector and REZEX 8μ organic acids column.

Results

Conditions of Hole 1301A. Data retrieved from borehole instruments and reported by *Wheat et al*. [2010] indicated that the temperature in the borehole rose steadily from ~4 ^{o}C to ~30 ^{o}C over the first three years of the experiment. During this time, bottom water was drawn down the borehole into the formation because the CORK observatory was not sealed. During this time, bottom water became slightly altered as it flowed down the borehole. At the beginning of the fourth year, flow down the hole stopped and overpressured formation fluid flowed into and up the borehole, causing the borehole temperature to rise significantly during a one week period (9/3/07-9/10/07) to ~64^{o}C, where it remained until our samples were recovered in August 2008. During this one-week temperature increase, the altered seawater was replaced with formation water that

has similar composition to borehole water from Baby Bare springs and nearby ODP site 1026B [*Cowen et al*., 2003]. OsmoSamplers placed in Hole 1301A provided a continuous record of borehole chemistry throughout the deployment [*Wheat et al*., 2010], showing that nitrate concentration decreased during the first two years from > 30 μM to near 0 μM, and ammonium increased in the last year from near 0 μM to around 800 μM [*Wheat et al*., 2010, *Orcutt et al*., 2010b].

Flow cell condition after recovery. Upon recovery of the instrument string in August 2008, all osmotically-driven pump housings were cracked, yet they still continued to function [*Wheat et al*., 2010]. Water flow through the chambers over the period of the experiment was confirmed by the distribution of the dark precipitate on the retaining sponges (Fig.3.2). Although we did not measure the pump rates directly, they were calibrated prior to deployment. Using the calibrated pump rate, 92 mL of borehole fluid (slightly altered seawater) was pumped through the flow cells during the initial three years of deployment. This volume was about 10 times that of the liquid in the eight chambers in an "*Experimental/Control*" pair, connecting tubes, and filter holder that were connected to each osmotic pump. During the final year of deployment, additional borehole fluids (hydrothermal waters) would have been pumped through the cells at a higher rate. In a parallel experiment in the same borehole, a maximum of 600 ml was pumped through a fluid sampler during the final year of the deployment [*Wheat et al*., 2010]. This faster pump rate is in part related to the increase in temperature and the alteration of the membrane as it aged in this warm, reducing environment. The 0.2 μm

filter membranes between "*Experimental*" and "*Control*" flow cells appeared to be intact, but may not have functioned with 100% efficiency.

Visualizing and counting cells on minerals. PFA-fixed cells from mineral surfaces, mineral washes, and removed fixative solution were visualized with epifluorescent microscopy. In all sample types, cells were attached to mineral particles and not free-floating. In general, mineral particles (either primary or secondary) harbored multiple cells (Figure 3.3A, B, C). The most common cell morphology was a rod shape, however many cells were ovoid or cocci. No filamentous organisms were observed in the fixed samples. Phase-contrast images of non-fixed mineral suspensions used to create microbial enrichments revealed a wide diversity of cell types, including large (~5 μM) ovoid gliding cells, rod-shaped microbes, and other presumed microbial structures or secondary minerals (Figure 3.3F).

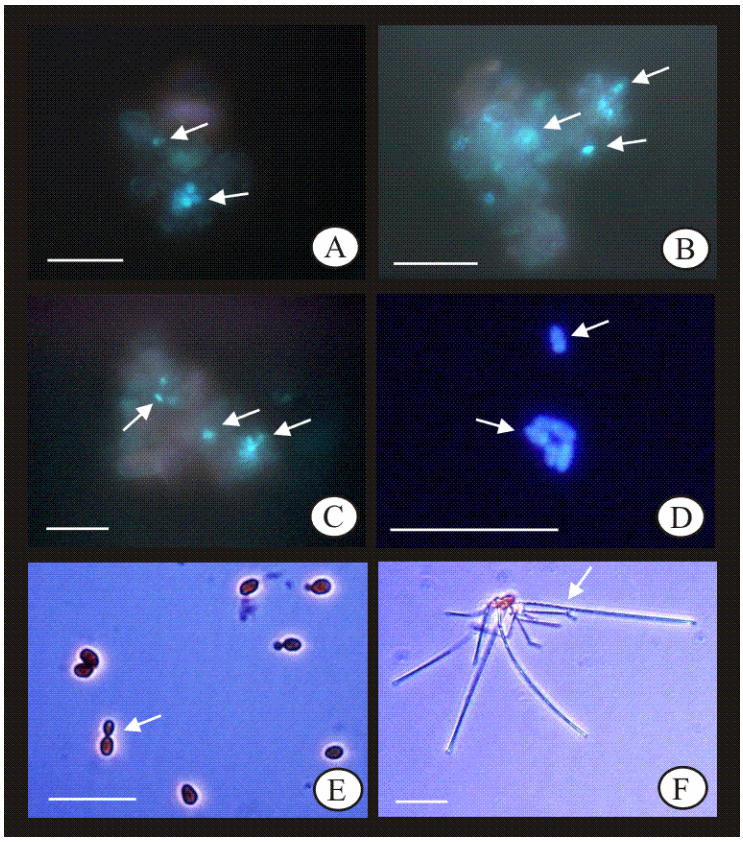

Figure 3.3. A-C) DAPI staining of PFA-fixed cells (arrows) attached to mineral particles from A) fayalite, B) olivine, and C) basalt. D) DAPI-stained cells visualized during cell counting from vortexed fayalite minerals. E) 1000X phase contrast images of marine oligotrophic broth culture of microfungi (*Rhodotorula sp*.) isolated from minerals incubated in Hole 1301A. Arrow indicates budding cells. F) 1000X phase contrast image of possible microbial structure in suspension of incubated basalt made with sterile seawater. These structures appear similar to twisted stalks (arrow) that are commonly produced by iron-oxidizing microbes. Bars equal 10μM.

Total cell counts by DAPI staining and microscopy (Table 3.2, Figure 3.3D) were achieved for both the "*Experimental*" and "*Control*" mineral groups. For each mineral specimen, the cell counts for "*Experimental*" minerals were always larger (2-25 times) than on "*Control*" minerals. Cell counts from the "*Experimental*" flow cells were highest

for the iron- bearing olivines (Fo_{90} olivine and fayalite, Table 3.2), with 45% of the total cells counted from all minerals originating from these two minerals.

Table 3.2. Total cell counts (from DAPI staining and microscopy) from minerals and glasses incubated in Hole 1301A. The ± values represent one standard deviation from triplicates.

Mineral/ Glass	Experimental flow cell chamber	Experimental Total cell counts (10^6 cells g-mineral^{-1})	Control flow cell chamber	Control Total cell counts (10^6 cells g-mineral^{-1})
forsterite	1A	140 ± 21	2A'	5.0 ± 1
olivine	1B	390 ± 19	2B'	39 ± 6
fayalite	1C	280 ± 48	2C'	19 ± 1
hornblende	1D	150 ± 2	2D'	60 ± 3
basalt	3A	88 ± 7	4A'	3.0 ± 1
obsidian	3B	92 ± 7	4B'	27 ± 2
augite	3C	91 ± 2	4C'	8.0 ± 2
diopside	3D	46 ± 11	4D'	27 ± 3
anorthite	5A	50 ± 14	6A'	6.0 ± 2
bytownite	5B	23 ± 4	6B'	3.0 ± 1
k-feldspar	5C	39 ± 10	6C'	12 ± 1
apatite	5D	100 ± 6	6D'	9.0 ± 0

Isolating and counting microorganisms. CFU counts for each mineral incubated in Hole 1301A show a significantly higher relative abundance of mesophilic oligotrophs (Table 3.3) on Fe(II)-containing minerals. Fo_{90} olivine and fayalite had the largest numbers of isolated oligotrophic mesophiles on mineral surfaces, with forsterite, apatite, and anorthite next highest in abundance. CFU counts for "*Experimental*" thermophilic aerobic oligotrophs (Table 3.3) showed that olivine and fayalite were the only minerals

containing these organisms, with trace amounts found on hornblende. The organotrophic oligotrophs (defined as microbes growing on low-organic media such as R2A) growing in mesophilic conditions were a very small fraction of the total microbial community (quantified by direct counting). On the other hand, the thermophilic oligotrophs were a larger fraction of the total community in olivine (25%) and fayalite (100%), and no thermophiles were found in non-olivine mineral chambers. No growth was observed in the 55°C iron dissimilatory enrichments over a 6 week period.

Table 3.3. The abundance of culturable oligotrophs (aerobes) from minerals and glasses incubated in "*Experimental*" flow cells 1, 3, and 5 from Hole 1301A.

Mineral/Glass	Abundance of mesophilic oligotrophs (10^6 CFU g-mineral^{-1})	Abundance of mesophilic oligotrophs relative to the total cell counts (in % ± 0.5%)	Abundance of thermophilic oligotrophs (10^6 CFU g-mineral^{-1})	Abundance of thermophilic oligotrophs relative to the total cell counts (in % ± 0.5%)
forsterite	6.0	4	0.0	0
olivine	8.0	2	95	25
fayalite	8.0	3	290	~100
hornblende	1.0	1	0.003	0.002
basalt glass	2.0	2	0.0	0
obsidian	3.0	3	0.0	0
augite	2.0	2	0.0	0
diopside	0.3	1	0.0	0
anorthite	4.0	9	0.0	0
bytownite	3.0	13	0.0	0
k-feldspar	2.0	6	0.0	0
apatite	5.0	1	0.0	0

Identification of aerobic oligotrophs. A total of 84 aerobic mesophilic oligotrophs isolated from all *"Experimental"* minerals were characterized by DNA sequencing. These sequences belonged to only 9 phylotypes (Table 3.4) distributed fairly homogenously across all minerals. The most commonly isolated organisms were related to the genera *Alcanivorax*, *Marinobacter* and *Halomonas*, all belonging to γ-*Proteobacteria.* *Marinobacter* isolates exhibited more than one colony morphology on MR2A simultaneously, indicating more than one type was isolated. Three isolates were most closely related to the yeast *Rhodotorula*. The DNA of *Rhodotorula* amplified with archaeal primers 4F and 1492R and was identified by 18S rDNA sequencing. The microfungi grew in colonies with pink pigmentation, cells were small (3-5 μm) and spherical to ovoid in shape, and exhibited budding (Figure 3.3E). α-*Proteobacteria* and Gram-positive *Actinobacteria* were also isolated in smaller numbers. A phylogenetic tree was constructed to show relatedness of isolated bacterial phylotypes to other known sequences or isolates (Figure 3.4). Sequences obtained from this study were submitted to GenBank under the accession numbers HQ621830, and HQ427421- HQ427430.

Table 3.4. Most common mesophilic organotrophic phylotypes isolated from flow cell minerals incubated in Hole 1301A. Habitats for 16S rDNA sequence relatives are included.

Phylogenetic group	Phylotype	Habitat of closest sequence relative
γ-Proteobacteria	*Alcanivorax*	Deep sea Mediterranean sediment
	Halomonas	Indian Ocean hydrothermal vent sediment
	Marinobacter	Ocean sediment
	Pseudomonas	Indian Ocean deep sea
α-Proteobacteria	*Aurantimonas*	Intertidal sand biofilms
	Nitratireductor	Deep Indian Ocean oil-contaminated water
	Stappia	Marine biofilms
high GC Gram+ Actinobacteria	*Microbacterium*	Indian Ocean deep seawater column
Urediniomycetes (fungi)	*Rhodotorula*	Bothnian Sea ice

The isolated thermophiles grew as very small round ivory colonies on MR2A media only. Gram staining revealed a large Gram-positive rod. We were able to retrieve only a small amount of biomass for DNA extraction and sequencing. Several attempts at amplifying the thermophile's DNA were unsuccessful with three pairs of 16S rDNA primers (8F or ITSF with 1492R for bacteria, and 4F and 1492R commonly used for archaea).

DAPI-stained thin sections. DAPI-stained thin sections of basalt glass and fayalite incubated in Hole 1301A (Figure 3.5) contained abundant cells in microfractures, however no clear evidence of bioweathering in association with these cells was found.

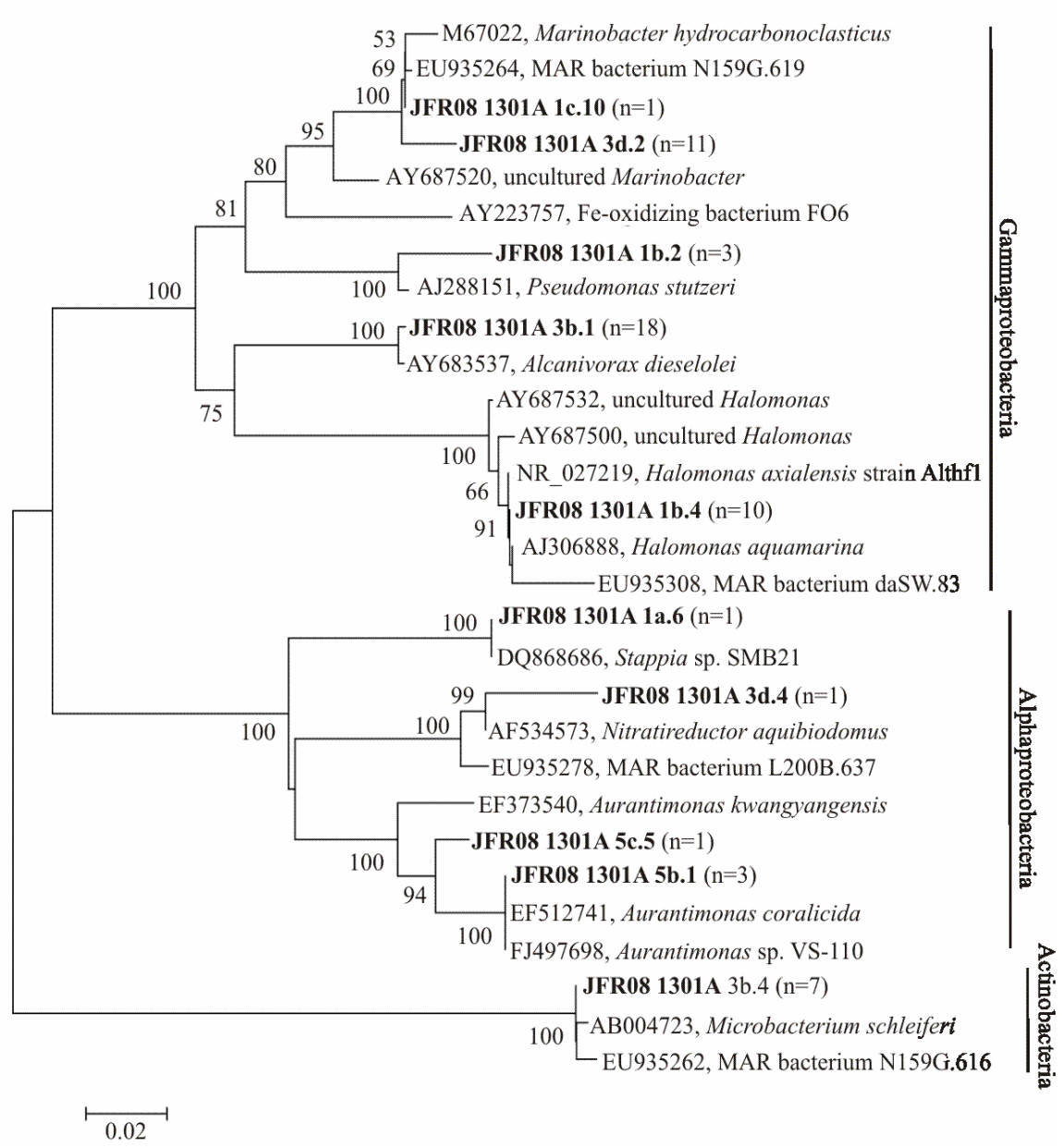

Figure 3.4. Phylogenetic analysis of isolates obtained from the minerals and glasses recovered from Hole 1301A in the JFR. Isolated organisms are shown in bold with the number of isolates obtained shown in parenthesis. Isolates were named with numbers corresponding to the flow cell number and letters corresponding to the mineral chamber (Table 3.1). The evolutionary history was inferred based on partial 16S rRNA gene sequences using neighbor-joining analysis. Bootstrap percentages above 50% (based on 500 replicates) are shown next to the branches. The sequence of *Aquifex pyrophilus* (M83548) was used as outgroup to root the tree (not shown). Bar, 2 substitutions per 100 nucleotides. MAR = Mid-Atlantic Ridge.

Physiological tests. All isolates are closely related to known nitrate reducers, and two isolates are related to known iron oxidizers (*Marinobacter* and *Pseudomonas*). *P. stutzeri* (the closest relative of the *Pseudomonas* isolates) and *Marinobacter* spp. have been shown to couple iron oxidation with nitrate reduction for growth [*Edwards et al*., 2003a; *Straub et al*., 1996]. We analyzed eleven strains, representing all nine identified phylotypes, for physiological characteristics relevant to growth on iron minerals. Testing for neutrophilic iron oxidation in $FeCO_3$ gradient tubes revealed discrete growth bands containing abundant cells (Figure 3.6). We also tested the eleven strains for dissimilatory nitrate reduction, nitrite reduction, and denitrification capabilities. Ten of the eleven strains tested were able to oxidize iron and reduce nitrate to nitrite, seven were able to further reduce nitrite, six were capable of denitrification to N_2 gas, and one could not perform any of the four physiologies tested (Table 3.5). Denitrification in nitrate broth was confirmed by the presence of gas in an inverted Durham tube, which was assumed to be N_2 gas since all gas-producing strains tested negative for carbohydrate fermentation [*Leboffe and Pierce*, 2005]. We included three strains of *Marinobacter* in our physiological testing because they appeared to be different strains based on colony morphology. *Marinobacter* strain 1c.10 grew only at or very near the abiotic redox boundary in $FeCO_3$ gradient tubes and was initially discounted as a potential irox oxidizer. This strain was determined to be a strict microaerophile when grown on MR2A gradient tubes containing resazurin as a redox indicator. Strain 1c.10 did not appear to reduce nitrate in nitrate reduction broth, however it was able to grow anaerobically on olivine in culture with nitrate as the oxidant and cysteine as a reducing agent (Figure

3.7A). More testing of this strain remains to be done to determine its full iron oxidation and nitrate reduction capabilities.

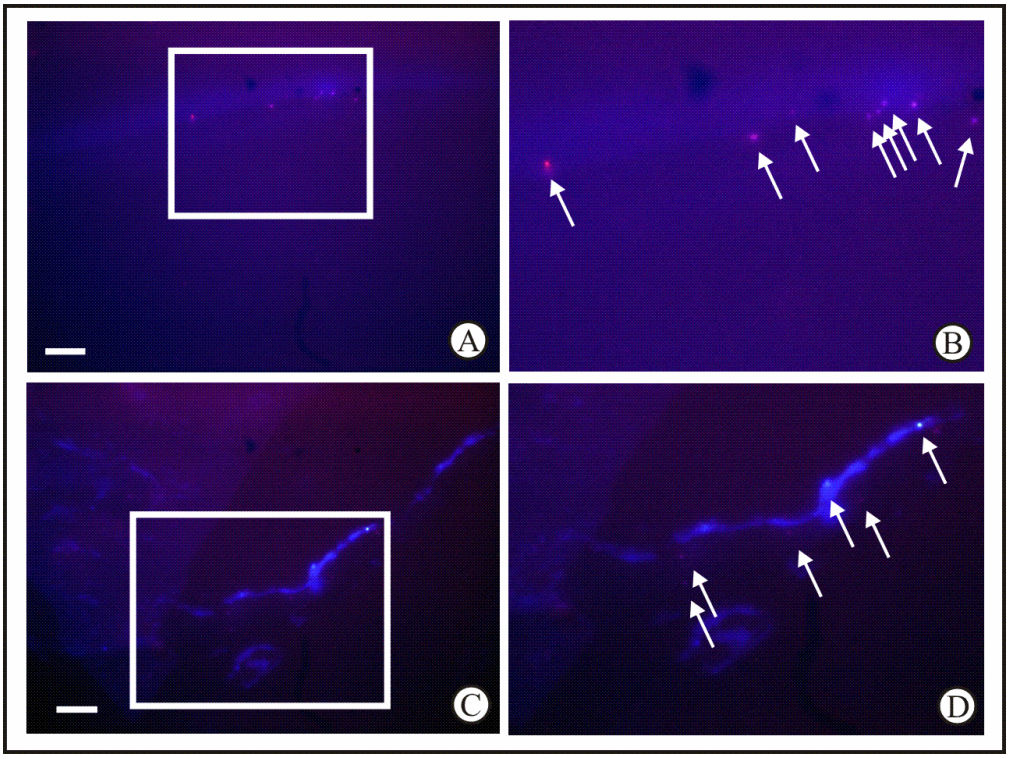

Figure 3.5. DAPI-stained thin sections (viewed with oil immersion at 1000x phase contrast microscopy) of olivine and basalt glass incubated in Hole 1301A. Cells are visible in microfractures (arrows). Inset from A) is magnified in B), and inset from C) is magnified in D). Bars are 10μm.

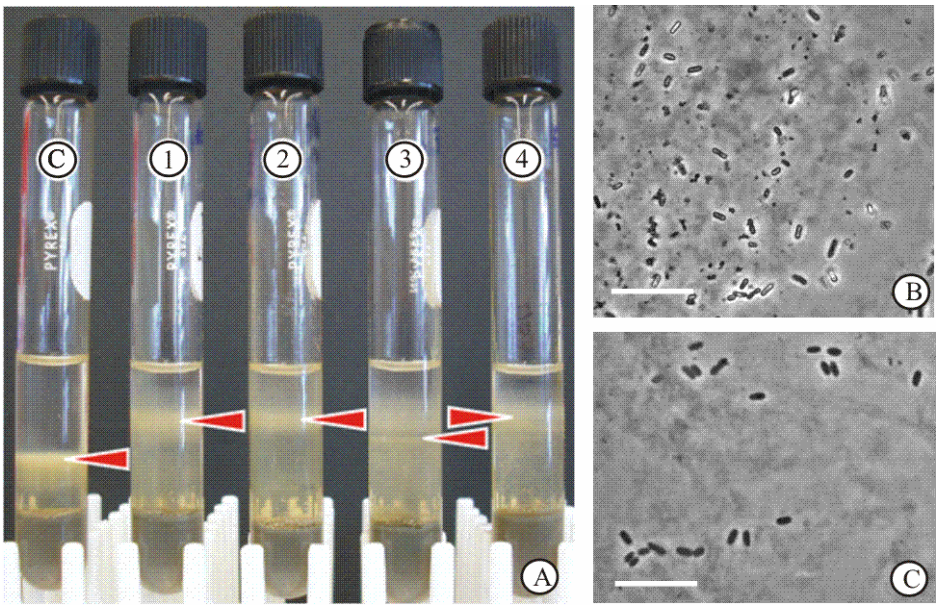

Figure 3.6. A) $O_2/FeCO_3$ gradient tubes incubated for 4 days at 25°C. Tube C is an uninoculated control; tubes 1 through 4 were inoculated with representative strains isolated from 1301A. Microbial growth is indicated by discrete bands containing abundant microbes (as evidenced by microscopy) and variable band position in the oxycline in relation to the control. Flags indicate the position of microbial growth bands in 1 through 4 and abiotic iron oxides in C. B) and C) 1000x phase contrast images of microbes sampled from growth bands in A). Bar equals 10μM.

Table 3.5. Results of physiological tests for eleven strains isolated from minerals and glasses incubated in Hole 1301A. + or – indicates "yes" or "no" for the ability to perform each physiological characteristic.

Strain name	Phylotype	Fe(II) oxidation	NO_3^- reduction	NO_2^- reduction	N_2 production (denitrification)
1c.9	*Alcanivorax*	+	+	+	+
5c.5	*Aurantimonas*	+	+	+	+
1c.1	*Halomonas*	+	+	-	-
1d.3	*Marinobacter*	+	+	+	+
1c.10	*Marinobacter*	+	-	-	-
3d.6	*Marinobacter*	+	+	+	+
1c.14	*Microbacterium*	+	+	+	+
3d.4	*Nitratireductor*	+	+	-	-
1b.2	*Pseudomonas*	+	+	+	-
5a.1	*Rhodotorula*	-	-	-	-
1a.6	*Stappia*	+	+	+	+

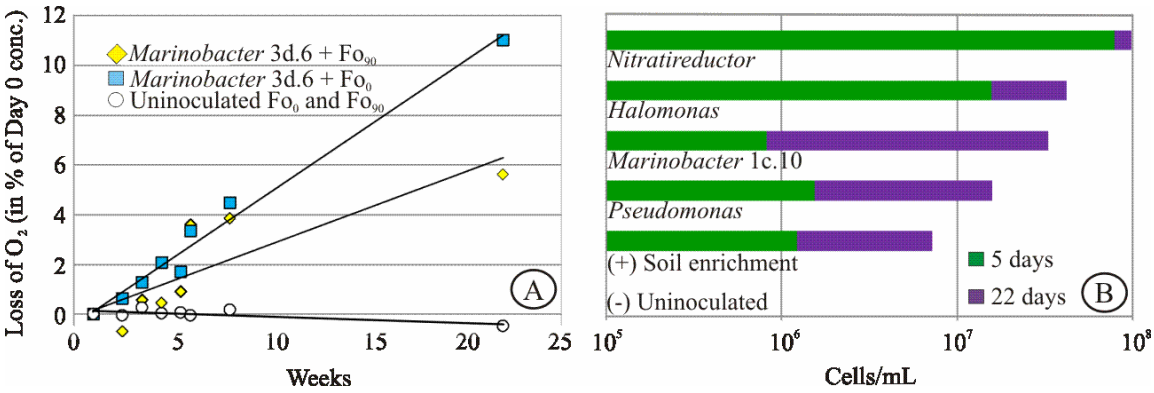

Figure 3.7. A) Growth of *Marinobacter* sp. 3d.6 as evidenced by O_2 loss over time when compared to uninoculated controls. Cells were grown with olivine containing 10% Fe(II) (Fo_{90}) or 100% Fe(II) (Fo_0) in marine mineral medium and 21% oxygen in the headspace. O_2 respiration is reported as loss of O_2 over a period of 22 weeks. B) Growth of four 1301A isolates in olivine media supplemented with 10mM. Included are an uninoculated negative control (-) and an organotroph aquarium soil enrichment as a positive control (+). No bar in the (-) indicates no growth. Growth (putatively indicating anaerobic nitrate-dependent iron oxidation using olivine) is reported as cells/mL over initial inoculum by cell counts on days 5 and 22.

Marinobacter strain 3d.6 consumed 5 – 11% of the available oxygen in 22 weeks of incubation in olivine medium with 21% O_2 in the headspace (Figure 3.7A). Growth by olivine oxidation is indicated by O_2 consumption with olivine as the only source of reducing power. Oxygen consumption in tubes containing fayalite (Fo_0) was twice as fast as in the olivine (Fo_{90}) tubes. Results from Fe(III)-reduction tests for strain 3d.6 showed no change in the amounts of lactate, acetate, succinate, or pyruvate over a 5 week period.

Four strains related to known iron oxidizers and nitrate reducers (*Nitratireductor*, *Halomonas*, *Marinobacter* sp. 1c.10, and *Pseudomonas*) were tested for the ability to grow on olivine utilizing nitrate as a terminal electron acceptor in the defined medium.

All four strains grew anaerobically on olivine using nitrate over the course of the 3-week experiment (Figure 3.7B).

Discussion

This is the first long-term deployment of microbial enrichment flow cells using osmotically-driven pumps in a subseafloor borehole. The three flow cell pairs worked as designed. This was confirmed by the numbers of microorganisms present in the minerals in the "*Experimental*" chambers compared to "*Control*" chambers. Flow through the chambers was also indicated by the pattern of dark and light foam plugs, presumably from iron sulfides (Fig. 3.2B). Circulation through the system was greater in the fourth year when the osmotic pump rate increased. The contamination of the "*Control*" flow cells could have been caused by compromised filters or seawater entering the flow cells through the broken pumps as the instrument string was hoisted to the ship. The lower number of cells in control minerals relative to experimental minerals indicates that the filters did work to some extent (Table 3.2). If seawater entered as backflow through the broken osmotic pumps, then chamber D' (which was closest to the pumps) should have been more heavily contaminated than the other control chambers. We did not find this to be the case.

Our initial goal was to isolate thermophilic iron oxidizers or iron reducers from the minerals incubated in Hole 1301A, based on our understanding that conditions at depth around Hole 1301A were similar to those around Hole 1026B (~64^{o}C, altered and reduced fluids) once downflow of bottom water into Hole 1301A stopped. Our

enrichments using olivine as a source of Fe(II) in low-oxygen autotrophic media did not produce any growth, and the same was true for our Fe(III)-reduction media. We also opted to isolate organotrophic organisms that could then be screened for the ability to oxidize iron as an alternate to autotrophic iron oxidizers [*Emerson et al*., 2010]. We were successful in this effort, yet much work remains to be done to link these particular organisms to iron oxidation and nitrate reduction in the ocean crust and to provide prove for chemolithoautotrophic growth.

The mesophiles we isolated are either thermotolerant remnants of the seawater microbial community that accumulated in the first three years (surviving 64^{o}C for ~1 year), or they originated from warm formation water and colonized the mineral surfaces during the final year of instrument deployment. The thermophiles may have originated from either formation water or seawater. The 84 colonies we sequenced clustered within only nine phylotypes, all closely related to known inhabitants of deep sea water, sediment, basalt, or hydrothermal deposits. Earlier researchers have identified microbes from seafloor basalts [*Mason et al*., 2009; *Santelli et al*., 2008; *Einen et al*., 2008; *Lysnes et al*. 2003, 2004], but in environments different from the one we studied. Most of the earlier work was done on seafloor basalts from cold, aerobic conditions, whereas Hole 1301A's environment changed drastically from cold and aerobic to warm and anaerobic during the last year of incubation. *Lysnes et al*. [2003] described a community of subsurface microorganisms in the Australian-Antarctic Discordance very similar to those found in Hole 1301A and cultured aerobic heterotrophs similar to those we have cultured from Hole 1301A. The phylotypes that were common between the *Lysnes et al*. study

and ours are *Pseudomonas*, *Marinobacter*, *Halomonas*, and *Actinobacteria*. *Lysnes et al.* [2004] reported *Pseudomonas, Actinobacteria*, and *Marinobacter* in Arctic seafloor basalt. Similar organisms were also isolated by *Rathsack et al*. [2009] from Mid-Atlantic Ridge (MAR) basalt, basalt glass, sediment, and seawater. These organisms include *Marinobacter*, *Halomonas*, *Pseudomonas*, *Microbacterium*, and *Nitratireductor*, all of which we have isolated from Hole 1301A. *Orcutt et al.* [2010b] found evidence suggesting that iron oxidizers were present in Hole 1301A during the low temperature period and that these microbial types were eliminated once the temperature increased. In support of this, we could not isolate any thermophilic iron oxidizers and iron reducers. However, *Orcutt et al.* [2010b] analyzed DNA sequences obtained from rock and mineral chips incubated in a simultaneous experiment in Hole 1301A and found that the majority of amplified sequences belonged to *Firmicutes*. Our isolates appear to be more indicative of inhabitants residing in cooler, more oxygenated regions of the surface and upper subsurface of the ocean crust than dominant members of the reducing hydrothermal environment in the deep subsurface.

All phylotypes we found are common in seafloor, subseafloor, or hydrothermal ecosystems. The *Halomonas* strain belongs to *Halomonas* Group 2A, which was previously identified as a subseafloor clade in JFR [*Kaye et al*., 2010]. The yeast belongs to a genus (*Rhodotorula*) with species ubiquitous in deep marine habitats [*Nagano et al*., 2010; *Connell et al.*, 2009; *Nagahama et al.*, 2006], and have been suggested to play a role in metal cycling in seafloor volcanic environments [*Connell et al*., 2009]. They were also found to be among the earliest colonizers of basalts in low-temperature seafloor

systems [*Connell et al*., 2009]. It is unlikely that our yeast isolate is a contaminant from seawater that may have entered the chambers of the flow cell during the string retrieval since all our other isolates are common to deep ocean crustal habitats. Moreover, *Rhodotorula* was abundantly common to most flow cell minerals, indicating its ubiquitous presence in the well water prior to the string retrieval. We propose that our isolate colonized the incubated Hole 1301A minerals during the first three years of the experiment when cooler borehole water was entering the flow cells. In summary, we think it is more likely that our isolates are representatives of the borehole fluids rather than contamination from the ocean column as the string was recovered.

The range of total cell densities we found (20 to 400 x 10^6 cells g-mineral^{-1}) are within values reported earlier for seafloor basalts [*Einen et al*., 2008; *Santelli et al*., 2008, 2009], the difference being that earlier reports analyzed densities in whole basalt rock, while our experiment was a flow-through enrichment on minerals and glasses. We found that the easily-weathered Fe(II)-bearing olivines contained the highest total cell densities, while the iron-poor minerals (diopside, anorthite, bytownite and K-feldspar) showed the lowest cell densities (Table 3.2). This is indicative of a microbial community potentially supported by neutrophilic iron oxidation. In addition, apatite (a phosphate-rich mineral) had cell densities comparable to forsterite, hornblende, obsidian and augite. This apatite enrichment is consistent with previous work, which indicated that microbes preferentially colonize apatite in phosphate-free media [*Rogers and Bennett*, 2004; *Bailey et al*., 2009].

Culturable mesophilic organotrophs were a small part (~1-13%) of the total microbial community. The largest densities of culturable organotrophs were found on the two iron-

bearing olivines, while most other minerals had smaller, similar cell abundances. Organotrophic thermophiles were only grown from minerals contained in flow cell #1, and were almost exclusively present in the Fo_{90} olivine and fayalite. The community of microbes from fayalite was almost 100 % represented by thermophiles. Finding more cells by culturing than by direct counting is likely explained by the presence of mature spores that escaped counting. Since spores stain poorly with the DAPI stain [*Setlow et al*., 2002], only the total number of vegetative cells were counted, and the total number of viable cells (including spores) was underestimated. Although we do not have DNA sequences for the thermophiles we have isolated, the cells stained as Gram positive organisms, which is indicative of *Firmicutes* (and spore-forming bacteria). *Orcutt el al*. [2010b] found putative thermophilic *Firmicutes* attached to the minerals they incubated in 1301A simultaneously with this study, and proposed that the thermophiles increased in abundance in the last year when the temperature increased. The thermophiles we have isolated may be representative of spore-forming organotrophs from this group of bacteria.

The structure of the microbial community very likely evolved during the four years of the experiment, but our experimental was not designed to monitor this evolution. The isolates selected from the marine R2A plates were similarly distributed across all minerals, yet this is not a comprehensive comparison of microbial community structure across different minerals since these isolates only represent a selected subset of culturable mesophiles. Our sampling focused on targeting a diversity of colony morphologies rather than random selection or the statistical relevance of each colony type.

All of our isolates are related to known nitrate dissimilators, while *Marinobacter spp*. and *P. stutzeri* (close relatives to two of our isolates) were shown to anaerobically oxidize iron with nitrate [*Straub et al*., 1996; *Edwards et al*., 2003a]. Our results are consistent with earlier observations that nitrate-reducing microbes may be common in the subseafloor of JFR [*Cowen et al*., 2003; *Wheat et al*., 2010]. A large majority of our mesophilic phylotypes were found to be nitrate reducers and iron oxidizers. These physiotypes may be common in seawater recharge zones where an influx of oxidants from seawater into subsurface basalt with Fe(II)-bearing minerals creates a chemical disequilibrium that can fuel microbial growth. However, we cannot deduce from these results that nitrate reducers and iron oxidizers are a dominant part of the subsurface community because the strains we analyzed were selected based on colony diversity and not randomly chosen.

Conclusions

Our goals were to test a novel, long-term, flow-through system for *in situ* mineral incubation in the ocean crust and to study the microbial colonization preference for minerals and glasses. We succeeded in isolating and culturing microbes from the upper ocean crust enriched *in situ* using igneous minerals and glasses as substrates. Samples were analyzed for total cell density and abundance of culturable organotrophs, and isolates were phylogenetically characterized. The highest total cell density and number of culturable organotrophs were found on Fe(II)-bearing olivines (Fo_0 and Fo_{90}). Of the twelve minerals and glasses analyzed, over 40% of the total cells were extracted from

only two minerals (Fo_0 and Fo_{90}). Among olivines, Fo_0 and Fo_{90} had significantly higher cell densities than the iron-poor olivine forsterite (Fo_{100}). Thermophilic organotrophs were only cultured from Fo_0 and Fo_{90}. We found that most culturable oligotrophs we have isolated from the igneous phases incubated in Hole 1301A are capable of growth by nitrate reduction and also have the ability to oxidize iron. Nitrate reduction *in situ* is supported by the observation that nitrate in the borehole was above 10 μM for the first two years and then declined [*Wheat et al*., 2010]. Our results suggest that oligotrophic microbes with ability to oxidize iron are an integral part of the microbial community residing in the upper basaltic crust of JFR. These physiotypes may also be common inhabitants of seawater recharge zones in subseafloor basalts containing olivine.

The *in situ* incubation of partitioned igneous minerals and glasses common in subseafloor basalt gives a unique insight into microbe-mineral interactions in the ocean crust. The development and implementation of novel techniques and devices (such as microbial flow cells) to evaluate subseafloor habitats and the ocean crust biome is vital to obtaining a comprehensive view of ocean crust microbiology. This study is the first quantitative analysis of microbial density associated with different minerals and mineraloids from ocean crust basalts. It is apparent that the association of microbes with minerals is controlled by mineral composition. This gives support to the hypothesis that endolithic microbial communities will have higher densities near minerals exhibiting greater thermodynamic disequilibrium with the surrounding fluid or seawater. It also proves that the heterogeneous distribution of minerals in marine igneous rocks will

directly influence the distribution of surface-attached microbial communities in ocean crust.

Acknowledgements

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CHAPTER 4

Olivine-oxidizing bacteria in Columbia River basalt-hosted systems (basalt rocks, river sediment, and soil)

Abstract

Olivine weathers quickly on geologic time scales, yet no iron-oxidizing microbes have been described that use the iron from olivine for energy in a natural habitat. This study is one of the first reports of successfully enriching for iron oxidizers by using olivine. Olivine oxidizers were isolated from Columbia River basalt-hosted terrestrial systems in Northern Oregon (basalt rock, river sediment, and soil). Isolates were tested for iron oxidation and nitrate reduction capabilities as well as the ability to grow on olivine. Four isolates were evaluated for bioweathering patterns produced on olivine and secondary mineral formation with X-ray diffraction. Isolates were phylogenetically related to known iron oxidizers, and all produced growth bands in the oxycline of $FeCO_3$ media. Each phylotype produced unique putative bioweathering patterns on olivine sands as visualized through light microscopy of petrographic thin sections. This is a significant finding since the ability of organisms to produce these features has never been demonstrated.

Introduction

The disequilibrium between oxidants and the Fe^{2+} from olivine can be utilized as a source of energy for microbial growth, however few reports of microbial growth

experiments using olivine exist [*Santelli et al*, 2001; *Welch and Banfield*, 2002; *Shirokova et al*., 2010; *Garcia et al*., 2005; *Longazo et al*., 2001, 2002; *Josef et al*., 2007]. Of these studies, only those by *Santelli et al*. and *Welch and Banfield* used olivine as the sole source of reducing power. Isolated organisms that were previously incubated in olivine-containing media in the laboratory are *Acidothiobacillus ferrooxidans*, an unidentified bacillus from a Columbia River basalt aquifer, a consortium of environmental microbes, *E. coli*, and a Pseudomonad. *A. ferrooxidans* is a well-known acidophilic iron oxidizer while Pseudomonads are heterotrophic bacteria whose lineage includes some organisms that are capable of neutrophilic iron oxidation [*Straub et al*., 1996]. Olivine has not been reported as being used successfully in enriching for iron oxidizers, therefore this study will be one of the first reports of successful enrichment for and isolation of iron-oxidizing bacteria using olivine in mineral media.

Columbia River flood basalts are a series of ~300 stacked basalt flows that erupted between 6–17 million years ago [*Alt and Hyndman*, 1995]. They are found in areas of Oregon, Washington, and Idaho and are as thick as 1830 m. Columbia River basalts dominate the Northern Oregon landscape and their microbial ecology is still being explored. *Stevens and McKinley* [1995] reported a subsurface ecosystem dominated by lithoautrotrophy and methanogenesis, however *Anderson et al.* [1998] disputed that the H_2 produced from basalt-water interactions was not enough to support a methanogenic population. *McKinley and Stevens* [2000] reported microfossils in Columbia River basalts, although the organisms that may have produced them remain elusive.

Iron oxidizers have been described from a variety of genera in *Bacteria* and *Archaea*. The most notable iron oxidizers are *Gallionella, Leptothrix, Mariprofundus, Ferroplasma, Ferroglobus, Spaerotilus, Sideroxydans, Acidothiobacillus, Leptospirillum, Acidovorax, Pseudomonas*, and *Marinobacter*. Many iron oxidizers are autotrophic, but many are heterotrophic and use iron oxidation as an alternative source of energy [*Emerson et al*., 2002, 2010; *Konhauser et al.,* 2011; *Edwards et al.*, 2003a,b, 2004; *Straub et al*., 1996]. Iron oxidizers are often acidophiles, since soluble Fe^{2+} is less susceptible to autooxidation at low pH. Neutrophilic iron oxidizers must compete with the O_2's strong oxidizing capacity, so they often prefer low O_2 conditions. Neutrophilic iron oxidizers are also known for producing sheaths and stalks as protection against free oxygen radicals or to prevent Fe^{3+} encrustation [*Emerson et al*., 2007]. Twisted stalk material is often the only evidence of iron oxidizers in iron mats [*Orcutt et al*., 2010b]. Neutrophilic iron oxidizers are common in basalts [*Stevens and McKinley*, 1995; *Emerson and Moyer*, 2002; *Edwards et al.*, 2003a,b; *Lehman et al*., 2004; *Emerson et al*., 2007; *Bailey et al*., 2009], but there are very limited reports of iron oxidizers that grow on olivine [*Santelli et al*, 2001; *Welch and Banfield*, 2002; *Shirokova et al*., 2010].

Materials and Methods

Sample collection. Three types of samples were collected for freshwater neutrophilic olivine oxidizer enrichments. Two samples of basalt were collected from the Willamette River in Portland, OR, sediment from the bank of the Sandy River in Troutdale, OR, and garden soil from Portland State University campus. The basalt samples were ground with

an alcohol and flame-sterilized mortar and pestle, and then 2 mL of sterile 0.2 micron-filtered H_20 was added to create a slurry. The slurry was transferred to a 2 mL microcentrifuge tube, and 100 μL was used to inoculate olivine culture media. Sediment and soil samples were suspended in 2 mL sterile H_20 by vortexing, and 100 μL was used to inoculate olivine culture media.

Olivine enrichments. Olivine enrichments were prepared in 10 mL screw-cap glass culture tubes using 3 ml of pH 6 Mineral Medium (MM). The MM contained: 20 mM Na_2SO_4; 1.5 mM KCl; 1 mM $NaHCO_3$; 0.5 mM NH_4Cl; 0.37 mM K_2HPO_4; and 20 mL/L Wolfe trace elements mix. The medium contained no vitamins to reduce heterotrophic growth. The Wolfe trace elements mixture contained: 12 mM Mg^{2+}; 12 mM SO_4^{2-}; 0.25 mM Mn^{2+}; 0.42 mM Co^{2+}; 0.68 mM Ca^{2+}; 0.37 mM Zn^{2+}; 0.118 mM Cu^{2+}; 21 μM Al^{3+}; 0.021 mM K^+; 0.16 mM BO_3^{3-}; and 41μM Mo^{6+}. Iron oxidizer enrichments contained 1 gram of Fo_{90} olivine as the reductant and 21% O_2 in the headspace as the oxidant. Tubes were inoculated with sediment, ground basalt, or soil. Serial dilutions were prepared from the original inoculated enrichments up to 10^{-5} and inoculated into new olivine MM. The highest dilution tubes containing evidence of growth were streaked for isolation on olivine agarose plates prepared with MM, ground olivine sand, and 1.2 % washed agarose. All enrichments, dilutions, and isolations were incubated at 30°C.

Organotroph isolation. After 1 week of growth, individual colonies were picked and inoculated onto new olivine MM plates to create a colony library. Colonies were also streaked onto Luria-Bertani (LB) medium to determine if the isolates were pure cultures

and if they were organotrophs. Pure colony cultures were used to extract DNA for phylogenetic analysis.

Phylogenetic analysis and tree construction for freshwater isolates. For phylotyping, the isolated colonies were scraped from plates and the genomic DNA (gDNA) was extracted in 99°C TE buffer for ten minutes [*Cook and Meyers*, 2003; *Mazza et al*., 2003]. The concentration of gDNA was quantified with a NanoDrop 1000 spectrophotometer. Supernatants containing DNA were used as a DNA template in a Polymerase Chain Reaction (PCR) targeting the 16S rRNA gene using either the bacterial 8F (5'AGAGTTTGATCCTGGCTCAG) or archaeal 4F (5'TCCGGTTGATCCTGGCRG) primers; and 1492R (5'GGTTACCTTGTTACGACTT) [*Baker et al*., 2003]. All PCR amplifications used a PCR kit (Fermentas) and a GeneAmp 2400 thermocycler (Perkin Elmer), and the following conditions (95°C for 3 minutes, 40 cycles of: 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, then a final 72°C for 5 minutes). The PCR products were verified by agarose gel electrophoresis, cleaned by using a PCR cleanup kit (Qiagen) and sequenced using an ABI 3130xl sequencer at the Oregon Health Sciences University DNA Core Facility.

Sequences using the 8F primer only were used for phylogenetic analysis. Sequences were imported into MEGA 4 and aligned with the nearest relatives. The evolutionary history was inferred using the Neighbor-Joining method [*Saitou and Nei*, 1987]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [*Felsenstein*, 1985]. The

evolutionary distances (base substitutions per site) were computed using the Maximum Composite Likelihood method [*Tamura et al*., 2004]. There were a total of 413 positions in the final dataset. Phylogenetic analysis was performed in Mega 4 [*Tamura et al*., 2007].

Limnobacter *growth on iron minerals.* Eight 50-ml culture jars were prepared with media containing a 1:100 dilution of R2A medium (DIFCO #218263) in 1:10 diluted mineral medium (MM). Table 4.1 shows how each culture jar was inoculated and the amount of reductant. A volume of 100 μL of MM-washed *Limnobacter* suspensions were used for inoculations. Cells were allowed to grow for 3 days at 30^{o} C, then 10 μL cell suspensions were stained with DAPI (see next section for procedure) and visualized at 1000x using epifluorescent microscopy. Relative cell abundances were reported as (-) = no growth, (+) = little growth, (++) = good growth, or (+++) = robust growth over the time period of the study.

Table 4.1. Experimental setup for iron oxidation growth study using *Limnobacter* sp. Sed5. Jar 1 was the uninoculated control to determine contamination, and jars 2 – 8 were all inoculated with a *Limnobacter* sp. Sed5 suspension. Each jar contained either no additional reductant (jar 2), or an iron or sulfate mineral/salt as the additional reductant. More growth in jars with iron (or sulfate) than in jar 2 would indicate use of the additional reductant for energetic growth.

Tube	Inoculated	Reductant
1	N	-
2	Y	-
3	Y	1g olivine
4	Y	1g pyrite
5	Y	10mM Na thiosulfate
6	Y	0.3g iron shavings
7	Y	0.7g steel brads
8	Y	100mM $FeSO_4$

DAPI staining of cells from iron media. Methods of counting cells on minerals from environmental samples were followed as described previously [*Bennett et al.,* 2006]. To obtain total cell counts, 10 μL aliquots of the vortexed serial dilutions were suspended in 90 μL of filter-sterilized 1x phosphate-buffered saline (PBS) buffer. Aliquots of this suspension (10 μL) were mixed with 90 μL of DAPI-PFA staining solution (5 μg/mL of 4', 6-diamidino-2-phenylindole solution in PBS with 4 % paraformaldehyde) and incubated for 5 min [*Bennett et al*., 2006]. One mL of PBS was added to each cell suspension and the mixtures were transferred to black polycarbonate 0.2 μm filters (Poretics), and washed with 2 mL of PBS buffer. Cells were visualized and counted using an epifluorescent microscope (Carl Zeiss, Axioscope).

Oxygen evolution in olivine media. Four freshwater isolates were inoculated into liquid MM containing 1 gram of olivine sand (Fo_{90}) prepared in 150 mL serum bottles that were sealed with 1 cm butyl stoppers under an excess pressure and air atmosphere. 100μL of each isolate was inoculated into the prepared medium and incubated at 30^{o}C for 60 days. O_2 and N_2 gas concentrations in the head space were measured by gas chromatography using an SRI 310C instrument, a Molecular sieve column, and a TCD detector. The gas pressure was measured with an Omega pressure meter (Omega Engineering, Inc. Ct).

Physiological tests. All phylotypes were evaluated for the ability to reduce nitrate and nitrite and to oxidize Fe(II) in $FeCO_3$ gradient tubes. For the nitrate reduction test, we prepared 10mM nitrate broth (DIFCO Catalog #226810) in culture tubes containing an inverted Durham tube to capture any gas produced from denitrification. After 4 days of incubation at 25^{o}C, cultures were analyzed for evidence of denitrification, and nitrate and

nitrite reduction as described previously [*Leboffe and Pierce*, 2005]. Strains that produced gas while growing in nitrate broth were also tested for carbohydrate fermentation using Triple Sugar Iron Agar [*Leboffe and Pierce*, 2005]. Negative testing for carbohydrate fermentation and gas production in the Durham tube of the nitrate broth was interpreted as positive for denitrification [*Leboffe and Pierce*, 2005]. Iron oxidation was verified using gradient tubes with a 30 mM $FeCO_3$ 2% agar plug at the bottom of the tube. The 0.5% soft agar overlay contained mineral medium (MM) supplemented with 30 mM NO_3^- as an alternate electron acceptor to O_2 and 2 mg/L resazurin redox indicator. Tubes were incubated at room temperature (25^oC) for four days. Iron oxidation was confirmed by the presence of a discrete growth band in the soft agar gel that was not present in the negative control. The increased abundance of microbes in the bands was verified by microscopy.

Petrographic thin sections. Samples were placed in sterile metal weigh dishes then 100 μL of DAPI solution (see ***DAPI staining of cells from iron media***) was added to each mineral sample. Samples were kept in the dark for all subsequent steps to avoid photo-degradation of DAPI stain. Samples were incubated at room temperature for 10 minutes, gently swirling a few times to mix. One volume of 95% ethanol was added to fix cells to minerals and incubated for 5 minutes. One volume of ddH_20 was added and swirled gently to mix, and then the liquid was decanted. Samples were rinsed with 5 volumes ddH_2O twice more and decanted again. A sterile pipette was used to remove any remaining excess liquid. Samples were allowed to dry in a warm, dark, oven overnight covered with aluminum foil. Petrographic thin sections of mineral sands were prepared

without heating at Oregon State University using Epo-Tek 301 low-fluorescence resin. Thin sections were viewed under light and fluorescent microscopy to visualize cells and search for mineral weathering patterns.

***X-ray diffraction*.** 5-month 30^{o} C incubated samples of freshwater strains in olivine MM (no vitamins or organics) were used for X-ray diffraction. 10 mL of sample medium with sloughing precipitate was filtered onto a 25mm 0.2μm nitrocellulose filter, and then washed with 20mL dd$H_2$0. Filters were placed on a glass slide while still wet and placed inside a Petri dish to avoid dust contamination. Samples were allowed to dry overnight at 35^{o} C. Samples were analyzed at Portland State University's Applied Mineralogical Laboratory on an X-ray diffractometer at ^{o}2θ. Sample peaks were compared to an olivine standard.

Results

Isolation and identification of olivine-loving bacteria from freshwater basalt, sediment, and soil. A total of 5 neutrophilic organisms were isolated from basalt rocks collected from the Willamette River in Portland, Oregon. However, phylogenetic analysis revealed they were all genetically similar and all clustered with *Delftia acidovorans*. Three isolates came from Sandy River sediment, two of which were from the bacterial genera *Acidovorax,* and one was most closely related to bacterial genera *Limnobacter*. The two soil isolates clustered with *Pseudomonas*. Isolates used in physiological studies are included in the phylogenetic tree (Figure 4.1).

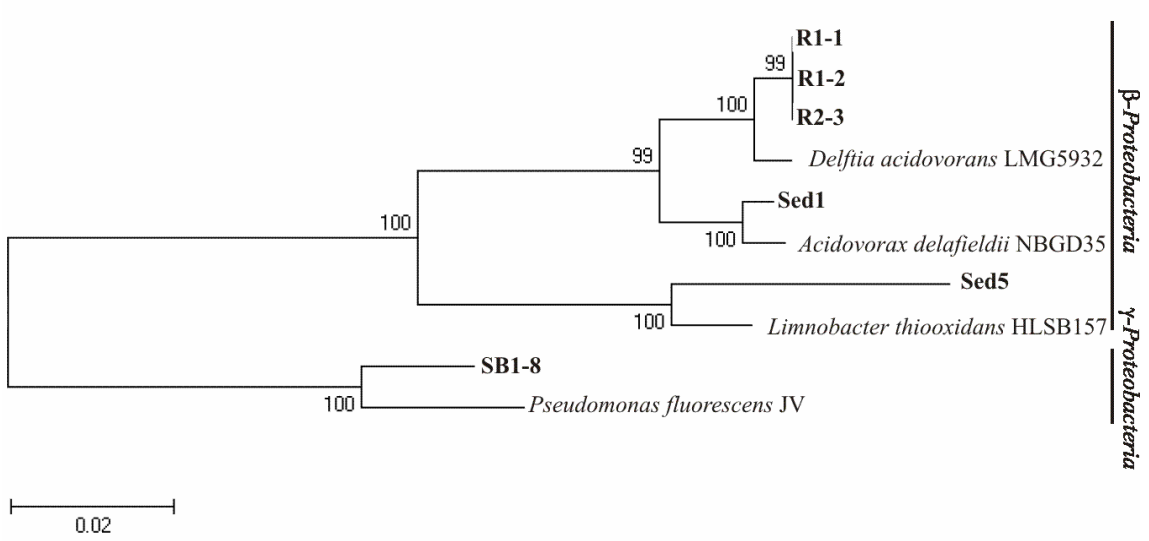

Figure 4.1. Freshwater isolate 16S rRNA gene phylogeny. Isolated organisms are bolded. Tree was constructed with Mega 4 using the closest relatives blasted in the NCBI database. Bar equals 2 substitutions per site. Bootstrap values (500 replicates) are placed at branch sites. A total of 413 nucleotides were used in the analysis. *E. coli* was used as outgroup.

Limnobacter *growth on iron minerals.* *Limnobacter* grew better on all sources of reduced iron than in oligotrophic conditions alone (Table 4.2). The growth of this strain also appears to be inhibited by the presence of Na-thiosulfate. Growth was highest on media containing sources of reduced iron including olivine, pyrite, iron shavings, and $FeSO_4$. Growth on steel brads was evident, but still less than olivine.

Oxygen evolution in olivine medium. All four freshwater isolates appear to use oxygen as a terminal electron acceptor in a redox reaction with Fe^{2+} from olivine. *Acidovorax* and *Pseudomonas* cultures lost twice as much oxygen than the uninoculated control over the 60-day period, *Delftia* lost 1.9 times the amount of oxygen, and *Limnobacter* lost 1.45 times the amount of oxygen when compared to the control. (Figure 4.2).

Table 4.2. Results of *Limnobacter* sp. Sed5 growth study in minimal organic medium supplemented with iron minerals or thiosulfate as reductants. Relative cell abundances are reported as (-) = no growth, (+) = little growth, (++) = good growth, or (+++) = robust growth over the time period of the study. N=No, Y=Yes.

Tube	Inoculated	Reductant	Growth
1	N	-	-
2	Y	-	+
3	Y	1g olivine	+++
4	Y	1g pyrite	+++
5	Y	10mM Na thiosulfate	-
6	Y	0.3g iron shavings	+++
7	Y	0.7g steel brads	++
8	Y	100mM FeSO4	+++

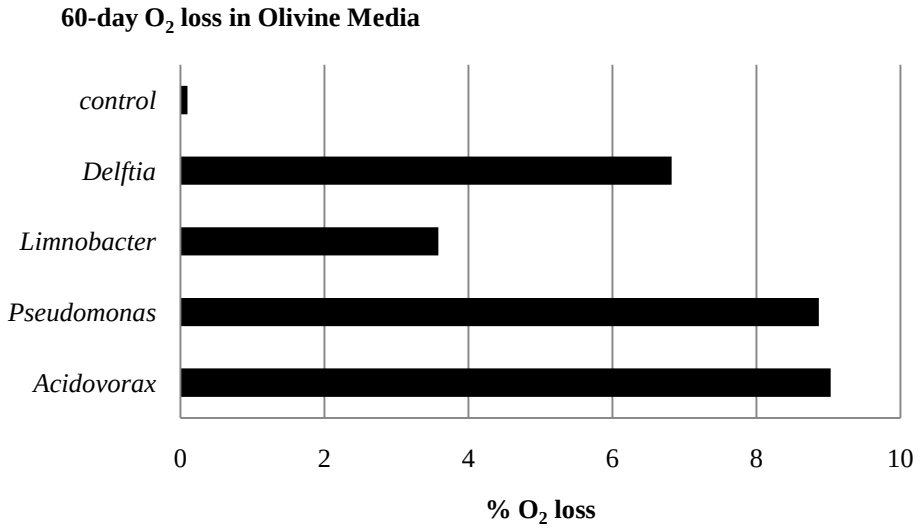

Figure 4.2. O_2 evolution over time in olivine media inoculated with four freshwater strains. Results are reported as percent oxygen loss (olivine oxidation) over a 60-day period, normalized to uninoculated control levels

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Physiological tests. In $FeCO_3$ gradient tubes, *Pseudomonas*, *Acidovorax*, and *Delftia* grew by iron oxidation as evidenced by a band of cell growth near the top of the oxycline. *Limnobacter*, *Acidovorax*, and *Delftia* were all capable of nitrate reduction, while

Pseudomonas was unable to reduce nitrate (Table 4.3). Only *Delftia* was able to reduce nitrate all the way to N_2 through denitrification.

Table 4.3. Physiological test results for freshwater strains isolated on olivine.

Phylotype	Fe(II) oxidation	NO_3^- reduction	NO_2^- reduction	N_2 production (denitrification)
Pseudomonas	+	-	-	-
Limnobacter	+	+	-	-
Acidovorax	+	+	-	-
Delftia	+	+	+	+

Petrographic thin sections. Petrographic thin sections were produced from olivine sand that had been incubated with each freshwater isolate for a 5-month period. A sterile negative control was also included for comparison. Results indicate putative biological weathering features were present on olivine, and each feature was unique to a particular isolate. No identifiable weathering features were seen in the sterile control (Figure 4.3A). *Delftia*-incubated olivine contained putative channels that were mostly perpendicular to the alteration front or microfracture (Figure 4.3B). *Limnobacter*-incubated olivine contained twisted and branched rust-colored tubular structures that permeated the minerals (Figure 4.3C, D). *Pseudomonas*-incubated olivine was quite different in appearance and showed an abundance of microscopic sand particles that accumulated in hollows and fractures (Figure 4.3E). *Acidovorax*-incubated olivine may have contained weathering features but they are weakly evident (Figure 4.3F).

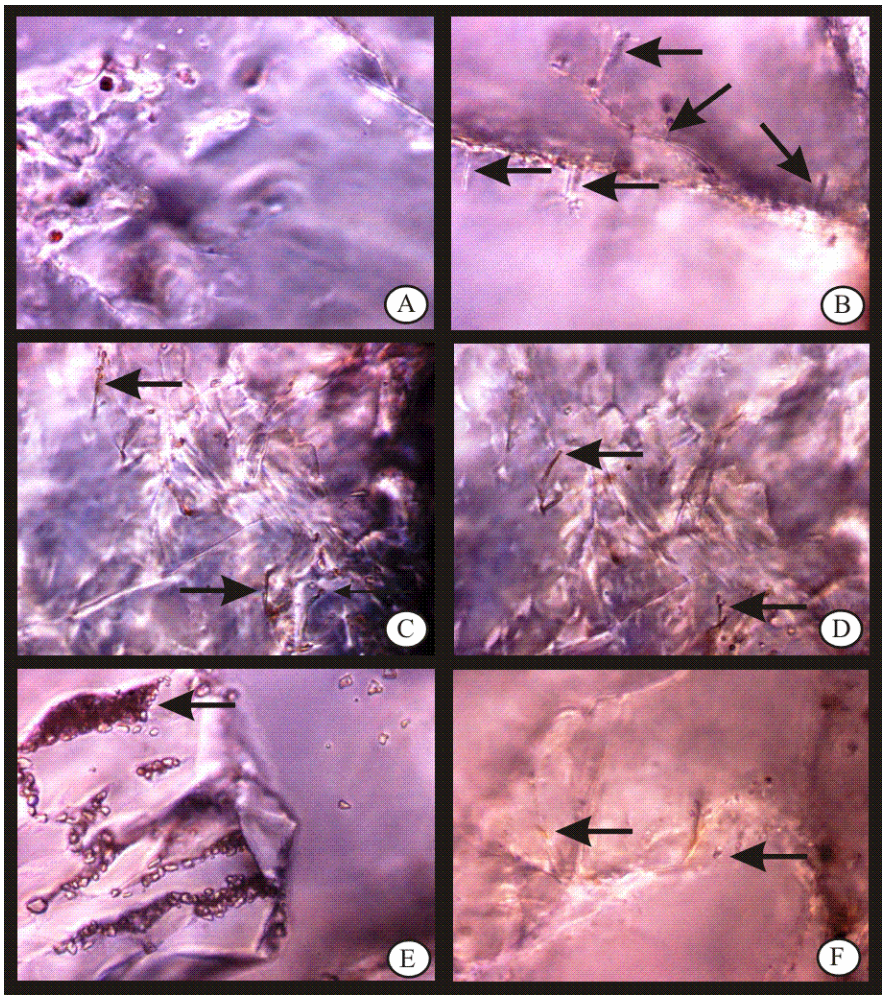

Figure 4.3. Petrographic thin sections of 5-month cultures containing MM, olivine sand, and A) no microbes, B) *Delftia* sp. R1-1, C) and D) *Limnobacter* sp. Sed5, E) *Pseudomonas* sp. SB1-8, and F) *Acidovorax* sp. Sed1. Arrows in B, C, and D point to tubular structures with an oxidized appearance. Arrow in E points to buildup of small grains of olivine in fracture areas. Arrows in F point to possible tubular structures. Tubular structures are approximately 1-2 microns in diameter and up to 10 microns long.

X-ray diffraction. The results of X-ray diffraction performed on the olivine "dust" from the 5-month incubated cultures all contain the same number of major peaks (5) at the same $^{o}2\theta$ (7.26, 5.08, 3.87, 3.70, 3.63, 3.48) (Figure 4.4). The sterile control diffraction pattern was very similar to the incubated sample patterns.

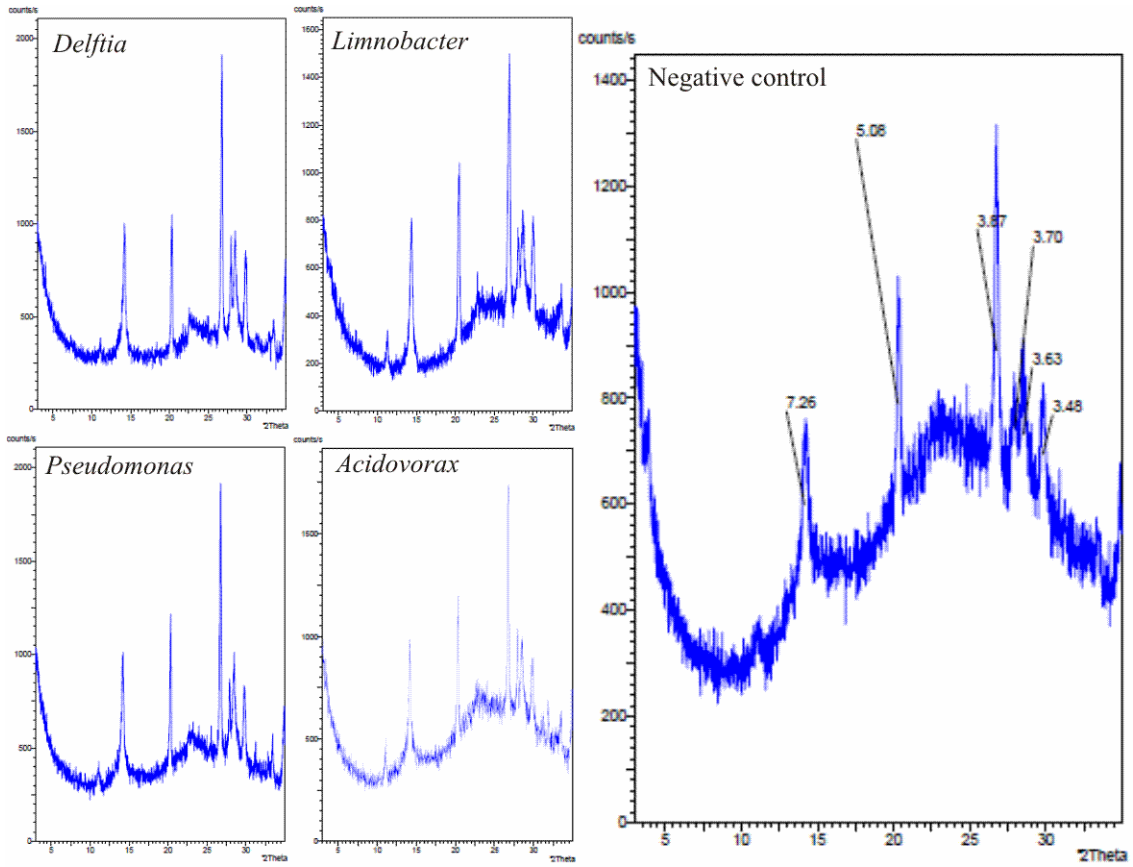

Figure 4.4. Results of XRD analysis of precipitates formed during 5-month incubation of freshwater strains with olivine in MM. Graph on the right is a sterile negative control.

Discussion

Olivine enrichments from freshwater igneous materials (soil, sediment, and basalt) originating from Columbia River Basalt deposits of northwestern Oregon produced a number of isolates that are closely related to known putative organotrophic iron oxidizers (*Pseudomonas*, *Acidovorax*) [*Straub et al*., 1996; *Pantke et al*., 2011], a thiosulfate oxidizer (*Limnobacter thiooxidans*) [*Spring et al.*, 2001], and an acetate-utilizing denitrifyer (*Delftia acidovorans*) [*Adav et al.*, 2010]. All four phylotypes appear to oxidize iron, three are nitrate reducers, and one is a denitrifyer (*Delftia*). All four

phylotypes grew in bands within the oxycline of $FeCO_3$ gradient tubes supplemented with nitrate as an alternative oxidant to atmospheric oxygen. All four isolates also consumed oxygen over a 60-day period in sealed bottles containing 21% O_2 in the gas phase, minimal mineral medium, and olivine sand. Since Fe^{2+} from olivine was the only reductant available, it is assumed that the oxygen consumption is due to iron-oxidizing activity. In addition, *Limnobacter* sp. Sed5 was tested for growth on a variety of iron and thiosulfate substrates as reductants, and this organism appears to grow better in media containing reduced iron than in media without it, including olivine. Interestingly, five-month cultures of each freshwater isolate with olivine sand may have produced biogenic alteration features in the olivine crystals as evidenced by light microscopy of petrographic thin sections. Also, precipitates in the media were subjected to X-ray diffraction, but even though precipitates increased in abundance over the incubation period, they do not appear to differ significantly from the parent material (olivine sand). This indicates there may be a possibility that microbial oxidation of iron increases the dissolution of olivine at neutral pH by increasing the rate at which Fe^{2+} is released from olivine in order to maintain equilibrium with reduced iron in solution. It could also indicate that any secondary minerals that were formed may not have been sufficient to be detected by X-ray diffraction. Either way, more studies are needed to determine if the release of fine olivine particles over time is biologically induced.

Conclusion

Bacteria isolated from olivine enrichments of basalt, river sediment, and soils are capable

of growing on olivine through iron oxidation. Isolated organisms are related to known iron oxidizers and appear to weather olivine in the process, although more study is needed. Putative microbial weathering features were produced in long-term cultures of isolates on olivine sand in mineral medium. This has implications for the study of biosignatures in igneous rocks which can be used in micropaleontology and astrobiology, and is a significant finding since no organisms have ever been shown to produce these features in a laboratory setting.

CHAPTER 5

Olivine-oxidizing Mars analogs isolated from Oregon lava tubes

Abstract

Lava tubes on Earth are cold, dark, oligotrophic environments that host abundant chemolithotrophic life. Lava tubes have been found on Mars and would represent an ideal safe harbor for life in near-surface environments. Life in Mars lava tubes would be sheltered from the harsh radiation and super-oxidizing conditions at the surface, yet the thermodynamic disequilibrium between oxidants in subsurface ice and reduced minerals could be used to generate energy for microbial growth. The boundary between ice and iron-rich basalt is a microenvironment that could support Mars-like organisms on Earth. Neutrophilic iron-oxidizing microorganisms from the basalt-ice interface in a lava tube from the Oregon Cascades with perennial ice were investigated. Nine phylotypes of bacteria were enriched for and isolated that are capable of growing in low temperature, oligotrophic conditions and utilizing Fe^{2+} for energy. Approximately 60% of the isolates belonged to two phylotypes, Gram-negative *Pseudomonas* and Gram-Positive *Flavobacterium*. *Flavobacterium* is a psychrophilic iron oxidizer and *Pseudomonas* sp. HerB is capable of growing on olivine. These two phylotypes are Mars analogs since their metabolic capabilities would allow them to live in the near-surface, icy, volcanic environments of Mars in the present or recent geological past.

Introduction

Lava tubes are basalt caves formed by underground lava flows that are often cold enough to house perennial ice. The interface between ice and basalt in such oligotrophic, aphotic environments are excellent analogues for Mars subsurface conditions. Life below the surface of Mars would be sheltered from intense radiation and superoxides, protected from temperature extremes, and have the advantage of a warmer and possibly wetter environment more conducive to life [*Fogg*, 1996; *Abramov and Kring*, 2005; *Travis et al*., 2003; *Clifford et al*., 2010; *Fairén et al*., 2010; *Samarkin et al.*, 2010].

Life at the basalt-ice interface in lava tubes has a physiology likely based on chemolithotrophic iron oxidation, since energy can be gained from the redox disequilibrium between oxidants in water or superoxides in surface dust, and reduced iron-rich basalt minerals such as olivine. Olivine $(Mg,Fe)_2SiO_4$ is a common mineral in basalt and is the most abundant mineral in the universe. The chemical signature of olivine can be seen in planetary disks, and it is abundant on planets such as Mars, in comet tails, and other rocky bodies where magma solidifies at a slow enough rate to allow for crystallization of minerals.

Iron oxidizers have been described from a variety of genera in *Bacteria* and *Archaea*, including *Gallionella*, *Leptothrix*, *Mariprofundus*, *Ferroplasma*, *Ferroglobus*, *Spaerotilus*, *Sideroxydans*, *Acidothiobacillus*, *Leptospirillum*, *Acidovorax*, *Pseudomonas*, and *Marinobacter*. Neutrophilic iron oxidizers must compete with O_2 for electrons, so they often prefer low-O_2 conditions. Neutrophilic iron oxidizers are common in basalts [*Stevens and McKinley*, 1995; *Emerson and Moyer*, 2002; *Edwards et al.*, 2003a,b;

Lehman et al., 2004; *Emerson et al*., 2007; *Bailey et al*., 2009], but there are very limited reports of iron oxidizers that grow on olivine [*Santelli et al*, 2001; *Welch and Banfield*, 2002; *Shirokova et al*., 2010]. This is the first report of olivine-oxidizing bacteria isolated from the basalt-ice interface in a lava tube cave with perennial ice. The presence of this physiotype in a Mars-analogue environment on Earth suggests that microorganisms could live in this microenvironment in the Martian subsurface, or may have lived in the Martian past when the temperature, atmospheric pressure, and perhaps the O_2 partial pressure were higher than today.

Materials and Methods

Collection and isolation of microbes from rock-ice interface. Rock and ice fragments were collected from high-elevation basalt lava tubes containing permanent ice in the summer of 2008. The samples were stored in sterile bags and packed in ice for transportation to the lab. Lava tubes that were sampled include South Ice Cave (Lat 43^{o} 34' 59''N, Long 121^{o} 04' 38''W), Arnold Ice Cave (Lat 43^{o} 54' 22''N, Long 121^{o} 09' 29''W), Surveyor's Ice Cave (Lat 43^{o} 38' 47''N, Long 121^{o} 15' 02''W), and Edison Ice Cave (Lat 43^{o} 55' 17''N, Long 121^{o} 36' 32''W), all in Deschutes National Forest, Oregon, USA. Arnold Ice Cave is now completely clogged with ice and its entrance is impenetrable, therefore only ice and rock from the cave mouth was collected for analysis. Rock and ice samples from Arnold Ice Cave and South Ice Cave were used in all microbial studies. Samples from Surveyor's Ice Cave and Edison Ice Cave were omitted from microbial studies due to warmer temperatures, influx of soils, and animal matter.

Enrichments for iron chemotrophic bacteria. Rock-ice fragments from South Ice Cave and Arnold Ice Cave were used to inoculate iron oxidizer growth media. Initial enrichment cultures contained 5 mL sterile 0.2 micron-filtered cave water (Arnold Ice Cave water for samples from AIC, and South Ice Cave water for samples from SIC) and one of three sources of iron: olivine sand (Fo_{90}), Fe_0 iron shavings, or Fe_0 in an agar plug to limit direct access by microbes. Serial dilutions of melted ice in contact in basalt were prepared and inoculated into the three types of iron media. The enrichment cultures were incubated at 5^o C for approximately 4 weeks. Psychrophilic or psychrotolerant bacteria were subsequently isolated from enrichments by streaking on R2A (Difco R2A agar Catalog #218263) low-organic plates to obtain pure cultures. Isolation using organic media was performed so that all iron oxidizers would also be organotrophs, which are more easily manipulated in the laboratory than autotrophic iron oxidizers. All samples and cultures were incubated between 0^o C and 10^o C to assure the isolates were cold-loving (psychrophilic) or psychrotolerant bacteria. Colonies exhibiting differing morphologies were saved in colony libraries and cells were frozen in sterile glycerol at -70^o C. All isolates were identified by 16S rRNA gene sequencing.

Phylogenetic analysis and tree construction for ice cave isolates. For phylotyping, the isolated colonies were scraped from plates and the genomic DNA (gDNA) was extracted in 99°C TE buffer for ten minutes [*Cook and Meyers*, 2003; *Mazza et al*., 2003]. The concentration of gDNA was quantified with a NanoDrop 1000 spectrophotometer. Supernatants containing DNA were used as a DNA template in a Polymerase Chain Reaction (PCR) targeting the 16S rRNA gene using either the bacterial 8F

(5'AGAGTTTGATCCTGGCTCAG) or archaeal 4F (5'TCCGGTTGATCCTGGCRG) primers; and 1492R (5'GGTTACCTTGTTACGACTT) [*Baker et al*., 2003]. All PCR amplifications used a PCR kit (Fermentas) and a GeneAmp 2400 thermocycler (Perkin Elmer), and the following conditions (95°C for 3 minutes, 40 cycles of: 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, then a final 72°C for 5 minutes). The PCR products were verified by agarose gel electrophoresis, cleaned by using a PCR cleanup kit (Qiagen) and sequenced using an ABI 3130xl sequencer at the Oregon Health Sciences University DNA Core Facility.

Sequences using the 8F primer only were used in phylogenetic analysis. Sequences were imported into MEGA 4 and aligned with the nearest relatives. The evolutionary history was inferred using the Neighbor-Joining method [*Saitou and Nei*, 1987]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [*Felsenstein*, 1985]. The evolutionary distances (base substitutions per site) were computed using the Maximum Composite Likelihood method [*Tamura et al*., 2004]. There were a total of 485 positions in the final dataset. Phylogenetic analysis was performed in Mega 4 [*Tamura et al*., 2007].

Physiological tests. All phylotypes were evaluated for the ability to oxidize Fe^{2+} in gradient tubes with a 30 mM $FeCO_3$ 2% agar plug at the bottom of the tube. The 0.5% soft agar overlay contained a mineral medium (MM) described below supplemented with 30 mM NO_3^- as an alternate electron acceptor and 2 mg/L resazurin redox indicator. The MM contained: 20 mM Na_2SO_4; 1.5 mM KCl; 1 mM $NaHCO_3$; 0.5 mM NH_4Cl; 0.37

mM K_2HPO_4; and 20 mL/L Wolfe trace elements mix. The medium contained no vitamins to eliminate possible organotrophic growth. The Wolfe trace elements mixture contained: 12 mM Mg^{2+}; 12 mM SO_4^{2-}; 0.25 mM Mn^{2+}; 0.42 mM Co^{2+}; 0.68 mM Ca^{2+}; 0.37 mM Zn^{2+}; 0.118 mM Cu^{2+}; 21 μM Al^{3+}; 0.021 mM K^{+}; 0.16 mM BO_3^{3-}; and 41μM Mo^{6+}. Tubes were incubated at 25° C for one week. To determine whether strains were psychrophiles or mesophiles, each strain was streaked onto R2A plates and incubated at 4° C or 25° C simultaneously. Psychrophiles grew better at 4° C.

***DAPI staining of* Pseudomonas sp. *HerB on cave rock thin sections*.**

Pseudomonas sp. HerB was inoculated in MM containing a petrographic thin section of South Ice Cave basalt and allowed to grow for 1 month at 30° C. Thin sections were removed with sterile forceps and allowed to air dry. Thin sections were then transferred to a 4% PFA-DAPI solution (5 μg/mL of 4', 6-diamidino-2-phenylindole solution in PBS with 4% paraformaldehyde) for 10 minutes. Stained samples were gently rinsed with 95% EtOH to adhere cells to the surface. Stained and fixed thin sections were then viewed under light and UV microscopy to visualize cells in relation to mineralogical features.

Results

Collection and isolation of microbes from rock-ice interface. South Ice Cave was most homologous with Martian cave environments (Table 5.1), and contained an abundance of clean (no visible organic matter) basalt and ice, indicating chemotrophs such as iron oxidizers would more likely be present. South Ice Cave had abundant microbial biofilms

on the surfaces of basalt on the cave floor, walls, and ceiling (Figure 5.1A, B, C), and an earthy smell. Basalt rock near ice or encased in ice had an oxidized appearance (Figure 5.1E).

Table 5.1. Sample parameters for Arnold and South Ice Caves.

Parameter	**Arnold Ice Cave**	**South Ice Cave**
Location of sampling	entrance	deep
Evidence of human contamination	+++	-
Organic debris	+++	-
Light	+++	-
Sample type	Melted ice containing basalt	Melted ice containing basalt
Mineral oxidation	+++	+++
Water source	Mouth of cave	Roof of cave

Enrichments for iron chemotrophic bacteria. Growth in enrichment tubes, although slow, was evident after one week of incubation at 3^{o} C. Cell growth was monitored by microscopy. Organotrophic R2A plates were inoculated with either Arnold or South Ice Cave enrichments containing olivine, Fe_0, or a Fe_0 plug as a source of redox energy for growth. Because iron oxidizes very quickly in neutrophilic conditions, it is possible that iron reducers were part of the enrichment in addition to iron oxidizers. After plating for organotrophic physiologies, a total of thirty two colony morphology-based isolates were chosen. Isolates were named and numbered consecutively according to original enrichment culture (*i.e.*, Ao4 is Arnold Ice Cave enrichment on olivine, colony morphology type #4). Three isolates were not successfully identified via sequencing and were omitted from analysis.

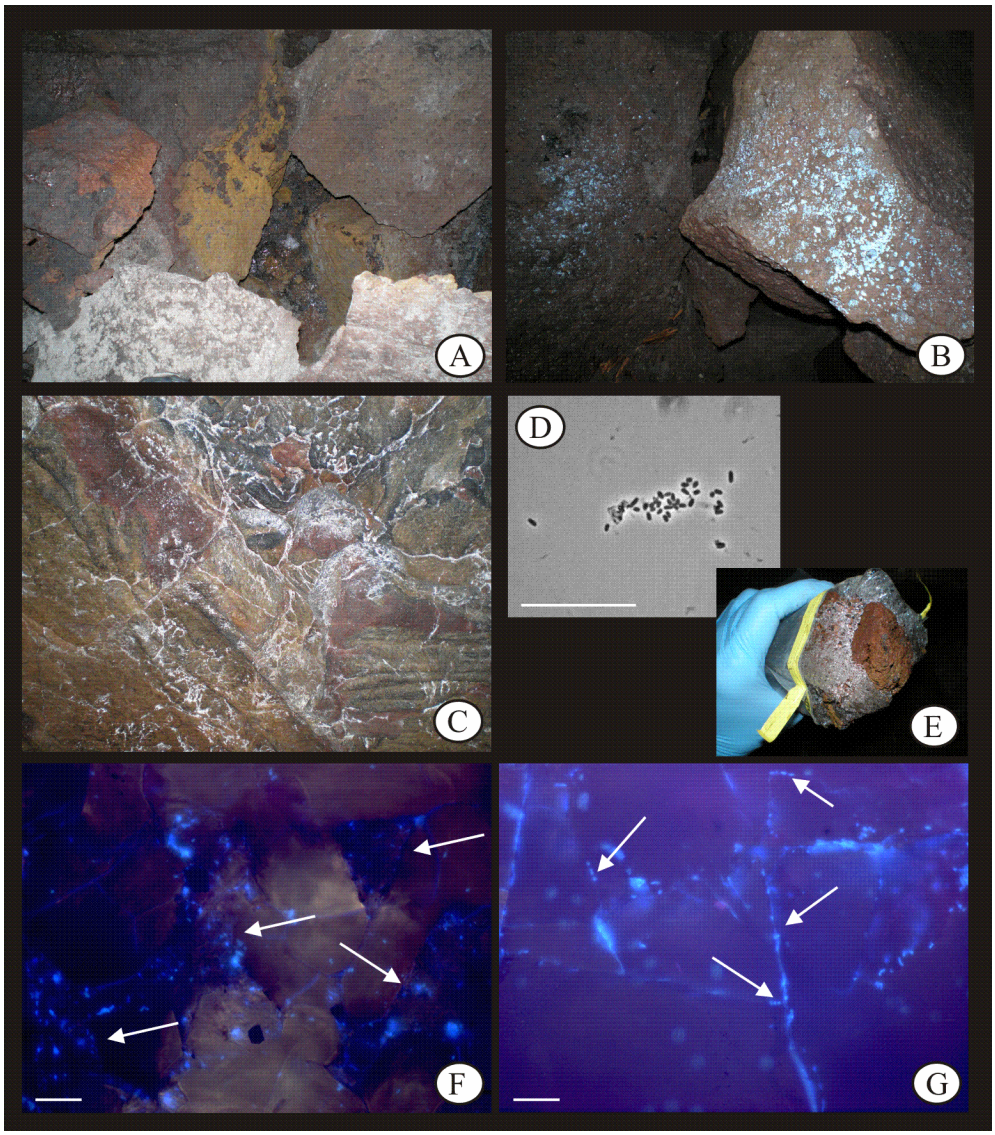

Figure 5.1. A – C) Microbial biofilms in South Ice Cave; D) *Pseudomonas sp.* HerB cells isolated from cave basalt growing on olivine; E) weathered basalt encased in ice from South Ice Cave; F – G) DAPI stain of *Pseudomonas sp.* HerB culture on ice cave basalt petrographic thin section. Arrows indicate cells concentrated on darker-colored minerals in F) and cells concentrated in microfractures in G). Bars equals 10 μm.

Phylogenetic analysis of ice cave isolates. All isolates from Arnold Ice Caves and South Ice Caves clustered within 9 distinct phylotypes. A phylogenetic tree containing all Gram-negative isolates (six organisms) and closely-related organisms is presented in

Figure 5.2. Figure 5.3 contains the Gram-positive ice cave isolates (three organisms) and related genera. Most isolates clustered with the genera *Pseudomonas* (~32%) and *Flavobacterium* (~29%) (Figure 5.4). Other isolated phylotypes include *Brevundimonas*, *Acidovorax*, *Dyadobacter*, *Acinetobacter*, *Pedobacter*, *Polaromonas*, and *Herminiimonas*. Approximately 65% of the isolates are *Proteobacteria*, 29% *Flavobacteria*, and 6% *Sphingobacteria*.

Physiological tests. Physiological test results of ice cave isolates are detailed in Table 5.2. *Flavobacterium* (and possibly *Polaromonas*) grew better at low temperatures than at mesophile temperatures while growing on organic medium. All other organisms are mesophilic organotrophs. Growth in $FeCO_3$ tubes was evident for the majority of isolates, including *Dyadobacter, Polaromonas, Herminiimonas, Flavobacterium, Pseudomonas, Acidovorax A* and *B, Pedobacter,* and *Rhodoferax. Dyadobacter, Acidovorax B,* and *Rhodoferax* grew very slowly in $FeCO_3$ medium (Table 10). *Dyadobacter* and *Polaromonas* produced very little biomass over the study period on R2A medium at 4^{o} C. *Flavobacterium* produced very little biomass in $FeCO_3$ medium, however this test was done at room temperature.

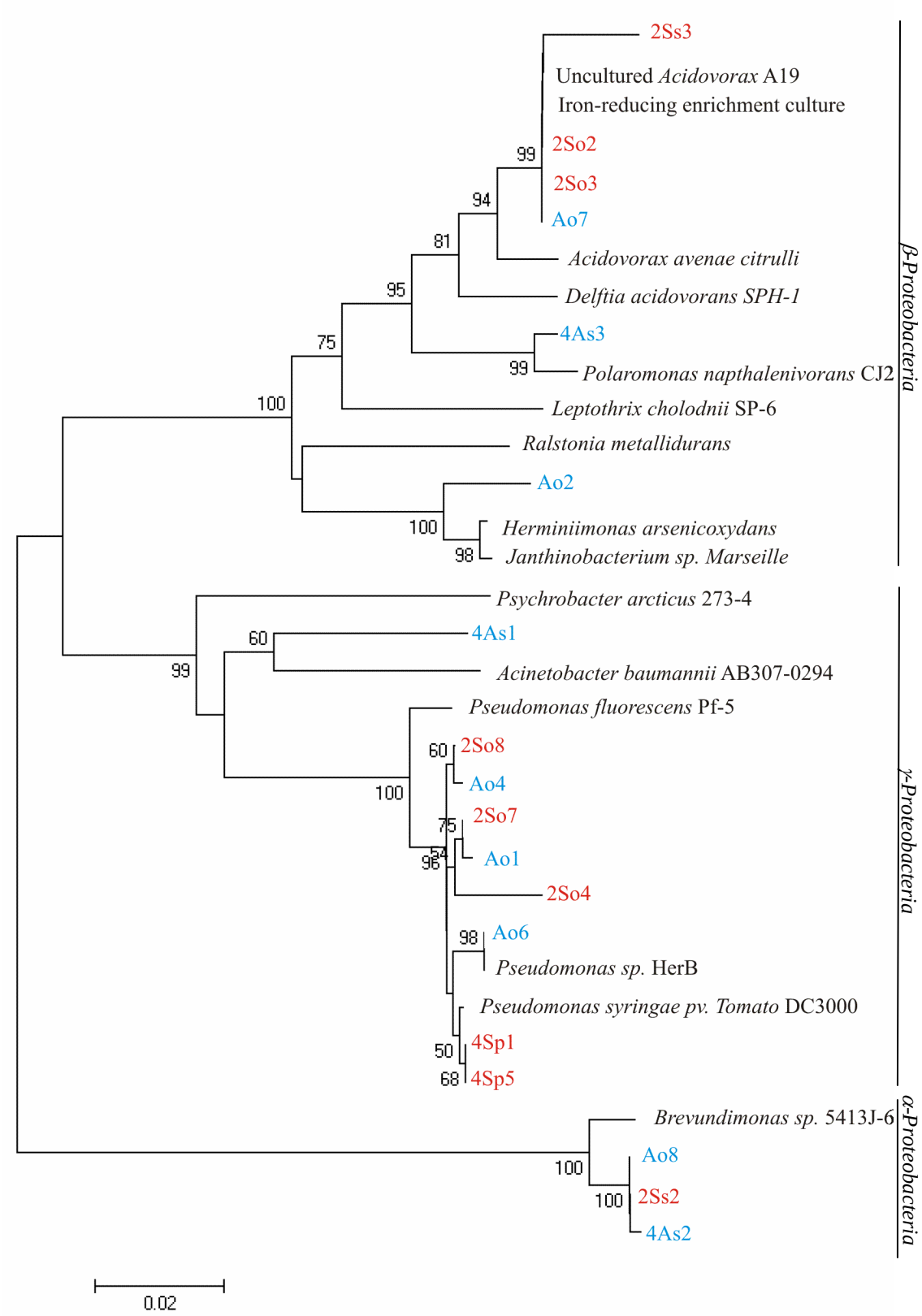
2Ss3
Uncultured *Acidovorax* A19
Iron-reducing enrichment culture
2So2
2So3
Ao7
Acidovorax avenae citrulli
Delftia acidovorans SPH-1
4As3
Polaromonas napthalenivorans CJ2
Leptothrix cholodnii SP-6
Ralstonia metallidurans
Ao2
Herminiimonas arsenicoxydans
Janthinobacterium sp. Marseille
Psychrobacter arcticus 273-4
4As1
Acinetobacter baumannii AB307-0294
Pseudomonas fluorescens Pf-5
2So8
Ao4
2So7
Ao1
2So4
Ao6
Pseudomonas sp. HerB
Pseudomonas syringae pv. Tomato DC3000
4Sp1
4Sp5
Brevundimonas sp. 5413J-6
Ao8
2Ss2
4As2
β-Proteobacteria
γ-Proteobacteria
α-Proteobacteria
0.02

Figure 5.2. Phylogenetic association of all Gram-negative isolates from Arnold (A - blue) and South (S - red) Ice Caves from enrichment media containing olivine (o), Fe_0 shavings (s), or an Fe_0 plug (p). The evolutionary history was inferred based on partial 16S rRNA gene sequences using neighbor-joining analysis. Bootstrap percentages above 50% (based on 500 replicates) are shown next to the branches. Bar, 5 substitutions per 100 nucleotides.

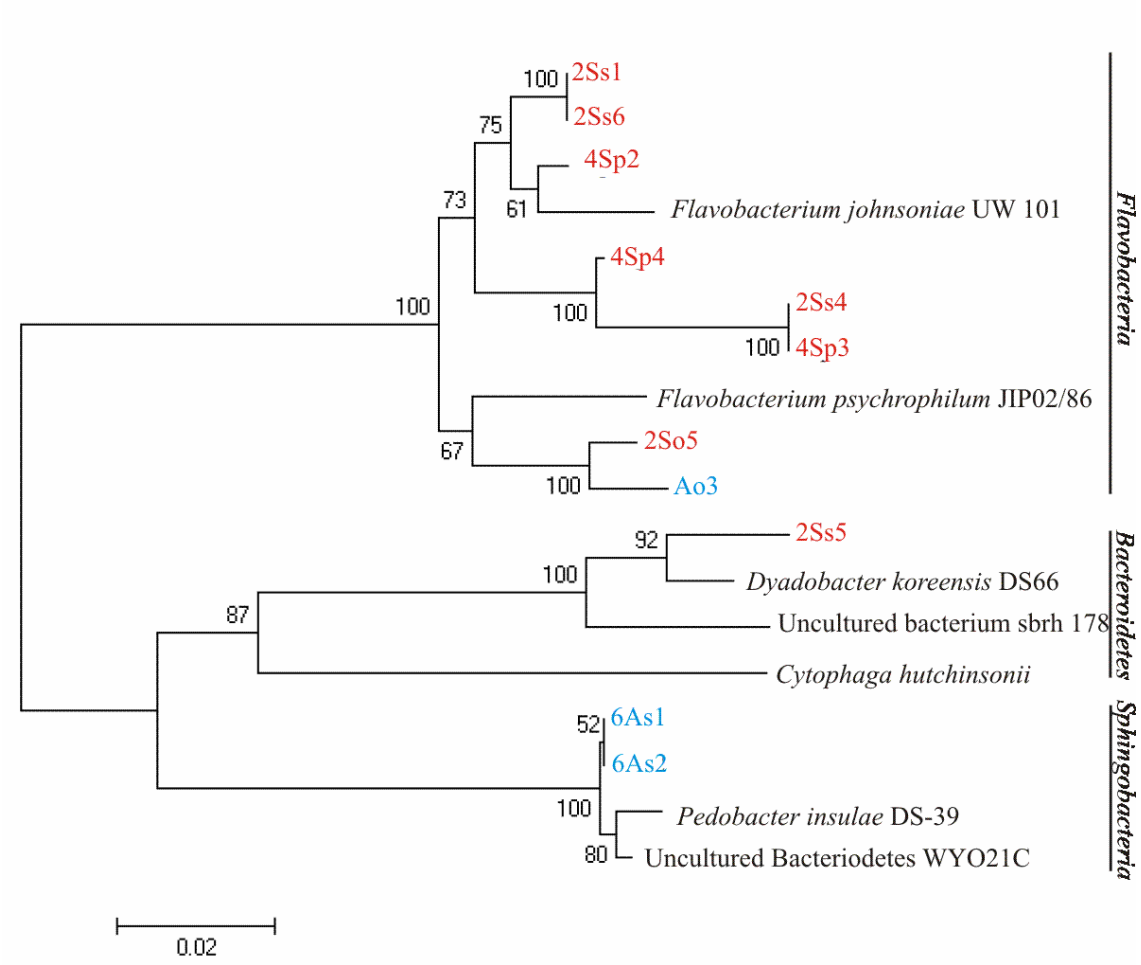

Figure 5.3. Phylogenetic association of all Gram-positive isolates from Arnold (A - blue) and South (S - red) Ice Caves from enrichment media containing olivine (o), Fe_0 shavings (s), or an Fe_0 plug (p). The evolutionary history was inferred based on partial 16S rRNA gene sequences using neighbor-joining analysis. Bootstrap percentages above 50% (based on 500 replicates) are shown next to the branches. Bar, 5 substitutions per 100 nucleotides.

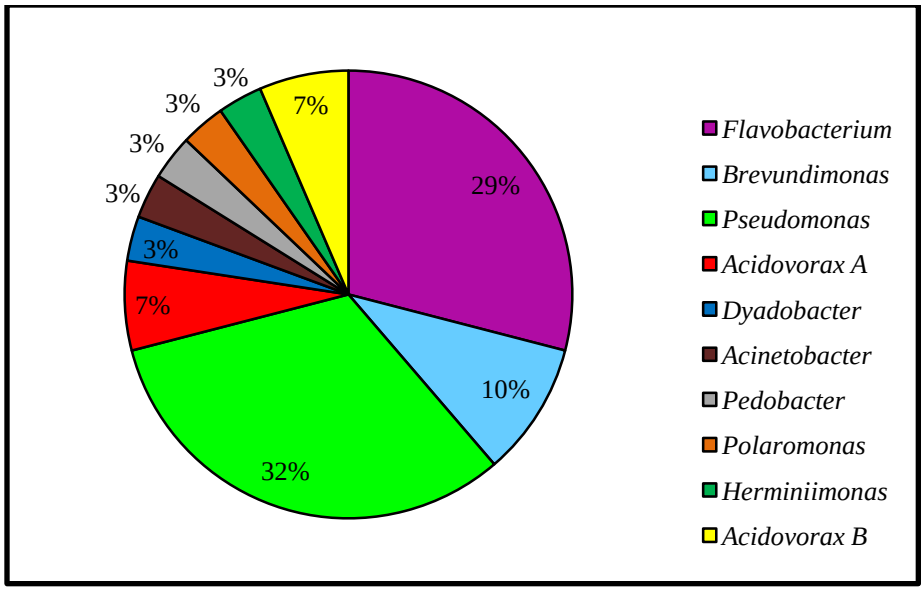

Figure 5.4. The frequency of isolates separated by genus. Organisms from the genera *Flavobacterium* and *Pseudomonas* were the most common bacteria isolated from the iron enrichment tubes.

Table 5.2. Physiological test results for ice cave isolates (4°C vs. 25°C and $FeCO_3$). (*) means very little biomass was produced, (s) means very slow growth observed.

Organism	Growth at 4°C	Growth at 25°C	$FeCO_3$ growth
Dyadobacter	+*	++	+,s
Polaromonas	+*	+	+
Herminiimonas	++	+++	+
Flavobacterium	++	+	+*
Pseudomonas	++	+++	+
Acinetobacter	+	++	-
Brevundimonas	+	++	-
Acidovorax A	+	++	+
Pedobacter	++	+++	+
Acidovorax B	++	+++	+,s
Rhodoferax	++	+++	+,s

DAPI staining of* Pseudomonas sp. *HerB on cave rock thin sections. Figure 5.1 panel D is a phase contrast image of *Pseudomonas sp.* HerB grown on olivine in the laboratory. Cells do not appear to have changed size or shape in response to the limited medium, and

appear similar to those cells grown in organic medium. Panels F and G in Figure 5.1 are images of DAPI-stained *Pseudomonas sp.* HerB cells grown on petrographic thin sections made of basalt from South Ice Cave. Cells are concentrated on specific mineral surfaces and in microfractures. Mineral surfaces where growth is abundant appear to be more weathered and contain abundant iron oxides (solely based on color of mineral grains).

Discussion

There was no significant difference in the variety of bacteria that were isolated between two ice lava caves in Oregon (South Ice Cave and Arnold Ice Cave), despite the varying environmental conditions. Many of the isolates are most closely related to known psychrophiles from Polar Regions and high altitude mountainous areas. There are slight differences in diversity between the two caves using the same culture methods for iron-oxidizing bacteria, although that is most likely due to sample location and other available energy sources. South Ice Cave contained numerous microbial biofilms, including what appeared to be iridescent colonies of *Actinomycetes* (Figure 5.1C) [*Northrup et al*., 1997], iron-manganese nodules, and others. The smell of geosmins, the "earthy smell" commonly produced by *Actinobacteria*, was prevalent inside the cave, which supports the assumption that *Actinomycetes* were present.

Twenty nine isolates were successfully amplified by 16S rRNA gene PCR, and they all clustered within 9 different phylotypes. Many of the closest relatives are from Polar and mountainous environments, which is interesting since the Oregon Ice Caves that were sampled are surrounded by hot, arid, high desert. It is possible these organisms, if

truly psychrophilic, were deposited during the last ice age and remained in the caves after the glaciers retreated. Six of the phylotypes are Gram negative, and three are Gram positive. Interestingly, four of the isolates are related to *Acidovorax* and *Delftia*, two of the isolates from Willamette River basalt and Sandy River sediment. The majority of the isolates (~32%) belong to the *Pseudomonas* group of Gram-negative bacteria. This is not surprising since Pseudomonads are well-known for their diverse metabolic capabilities and large number of species. Approximately 29% of the isolates belong to the Gram-positive *Flavobacteria.* The *Flavobacterium* phylotype (and possibly *Polaromonas*) grew better on organic medium at 4^{o} C than at 25^{o} C. These two organisms also grew very slowly and produced little biomass. These characteristics may indicate that these two organisms are more likely to be natural residents of the lava tubes than transients. Another interesting feature of these two isolates is that they grew in $FeCO_3$ medium, indicating putative iron oxidation capabilities. *Pseudomonas* sp. HerB was shown to grow on olivine that was charred to remove residual organics (see APPENDIX), which indicates this was not opportunistic growth, but growth based on iron oxidation using olivine. All of the isolated phylotypes grew in cold temperatures, although most grew slower than at room temperature on organic media. All isolates except for *Acinetobacter* and *Brevundimonas* grew in the $FeCO_3$ gradient tubes, indicating that iron oxidation may be an important metabolic feature of lava tube microbes.

DAPI staining of ice cave isolate *Pseudomonas* sp. HerB growing on a thin section of cave basalt indicated that some minerals were energetically more favorable to be attached to. Those mineral surfaces with greater abundance of cells were more oxidized, which

may be an indication that this isolate prefers iron-rich minerals or glasses over iron-poor phases. More work remains to determine the exact nature of the relationship between ice cave microbes and lava tube basalts.

Conclusion

The enrichement and identification of a number of bacteria from lava tubes in Oregon by enriching with olivine and Fe^{2+} was successful. Many of the isolates are putatively capable of oxidizing iron, and *Pseudomonas* sp. HerB has been shown to use the iron from olivine for growth in culture (see APPENDIX). This work is a preliminary indication that resident microbial populations may value iron oxidation as a part of their metabolic repertoire in order to survive in this oligotrophic and energy-limited environment. Further, many of these microbes, *Flavobacterium* and *Pseudomonas* sp. HerB in particular, would be excellent analogues for Mars subsurface life forms and their study would contribute greatly to the astrobiology community.

SUMMARY

This thesis provides the first comprehensive look at olivine-oxidizing microbes in the igneous subsurface. The majority of bacteria isolated and counted from deep ocean crust-incubated igneous minerals were attached to olivine minerals, and most of those phylotypes were capable of iron oxidation and nitrate reduction in the laboratory. A total of nine phylotypes of organotrophic bacteria were isolated, all related to known ocean crust, deep subsurface, and hydrothermal environments. At least four bacterial phylotypes were enriched on olivine media and isolated from soils, sediments, and basalt from Columbia River Basalt-hosted systems. These organisms were also capable of oxidizing iron, and three were nitrate reducers. All organisms grew on olivine and consumed oxygen in organic-free medium, indicating these organisms are capable of olivine oxidation. Isolates from nine bacterial genera were also enriched for and isolated using Fe^{2+} media (including olivine) from South Ice Cave in the Oregon Cascades. At least one organism is capable of growing on olivine, *Pseudomonas sp. HerB*, and one is a psychrophile (*Flavobacterium*). Interestingly, *Pseudomonas* was cultured from all three major environmental types, demonstrating the ubiquity and metabolic diversity of this genus.

The hypotheses were all strongly supported by data presented in this thesis, except for Hyp 3. Future work will investigate the relationship between community structure of individual minerals in the deep ocean crust and mineral composition by applying high-thoughput sequencing to the minerals incubated in Hole 1301A in the JFR.

Hyp 1. Mineral heterogeneity influences microbial distributions in subseafloor basalts (*i.e.*, total microbial abundances will vary according to mineral).

Supporting result: Olivine and fayalite were more heavily colonized in the deep ocean subsurface than other igneous minerals and glasses. Since basalts are mineralogically heterogeneous on the 0.01 to 10 millimeter scale, microbial populations are expected to be unevenly distributed in subseafloor basalts.

Hyp 2. Energy-rich minerals (such as olivine) will host a greater abundance of life than energy-poor minerals.

Supporting result: This work shows that energy-rich minerals (olivine and fayalite in particular) hosted a greater abundance of both total cells and culturable organotrophs than other minerals and glasses in the ocean crust. The same is presumed to occur in the terrestrial subsurface. The increased abundance of microbes on olivines, the minerals containing the highest percentage of redox-active elements combined with ease of weathering, supports this hypothesis.

Hyp 3. Community structure of attached microbes will vary according to source mineral (*i.e.*, iron-rich minerals will host different microbial communities than iron-poor minerals; iron oxidizers will be more prevalent on iron-rich mineral surfaces).

Supporting result: Due to the constraints of the subseafloor basalt study, we can neither confirm nor deny this hypothesis. Additional phylogenetic and molecular studies are required to give support to this hypothesis. Future studies include high-thoughput sequencing of DNA extracted from each incubated mineral

incubated in the subseafloor. The result of this study will give us a better indication of community structure and how it relates to mineral content.

Hyp 4. Microbes attached to olivine in surface and subsurface environments are capable of iron oxidation using oxygen or nitrate as terminal electron acceptors.

Supporting result: This was found to be true for all environments that were investigated. Iron oxidation may be a common metabolic capability of attached microbes living in the igneous subsurface. Interestingly, many of the microbes could also use nitrate as a terminal electron acceptor.

Hyp 5. Subsurface psychrophilic iron-oxidizing microbes are found on Earth today and are physiologically capable of living on Mars today or in the past when liquid water was present.

Supporting result: This work shows that *Pseudomonas* sp. HerB is capable of growing on olivine, *Flavobacterium* is a psychrophilic iron oxidizer, and many other phylotypes from lava tubes with perennial ice could serve as Mars analogues.

To address the primary questions of this thesis, the microbial colonization preferences for igneous minerals and glasses in the subseafloor are for energy-rich olivines and phosphate-rich apatite. Iron oxidizers are living on olivine surfaces in nature, and microbes that can grow on olivine can be isolated from a wide variety of environments. Psychrophilic iron oxidizers are living in Mars-analogous environments on Earth (such as ice lava tube basalt), and would be excellent for the study of life on other planets. In summary, iron-oxidizing bacteria are a dominant part of the microbial ecology of olivine

minerals, there are a variety of organisms that attach to and grow on olivine, and olivine distribution in the Earth's crust may influence microbial distributions in the subsurface.

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APPENDIX

Olivine-Respiring Bacteria Living in Mars-Like Conditions at the Rock-Ice Interface in a Lava Tube Ice Cave

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Abstract

The boundary between ice and basalt on Earth is an analog for some near-surface environments of Mars. We investigated neutrophilic iron-oxidizing microorganisms from the basalt-ice interface in a lava tube from the Oregon Cascades with perennial ice. One of the isolates (*Pseudomonas sp. HerB*) can use ferrous iron Fe(II) from the igneous mineral olivine as an electron donor and O_2 as an electron acceptor. Bicarbonate is a facultative source of carbon. The optimum growth temperature is ~12-14^{o}C, but growth also occurs at 5^{o}C. Growth of *Pseudomonas sp. HerB* as a chemolithotrophic iron oxidizer with olivine as the source of energy is favored in low O_2 conditions (*e.g.*, 1.6 % O_2). Most likely, microbial oxidation of olivine near pH 7 requires low O_2 to offset the abiotic oxidation of iron. The metabolic capabilities of this bacterium would allow it to live in near-surface, icy, volcanic environments of Mars in the present or recent geological past, and make this type of physiology a prime candidate in the search for life on Mars.

Introduction

The present day temperature of Mars' surface is mostly below the freezing point of water and the thin atmosphere leaves the surface exposed to cosmic and solar radiation. Because of inhospitable conditions, primary production through photosynthesis is assumed not to occur. Yet, the shallow subsurface of the Red Planet where temperatures are above freezing, could harbor chemolitho- autotrophic microorganisms. In the recent geological past Mars' surface could have been above freezing because of residual

geothermal heat, orbital forcing, or greenhouse gas effects (Carr, 1996; Fogg, 1996; Abramov and Kring, 2005). Liquid water could have existed on Mars over much of the planet's history, and may still exist at depth, at the rock-ice interface, inside rocks and soil, after impact events, and in brines (Travis *et al*., 2003; Clifford *et al*., 2010; Fairén *et al*., 2010; Samarkin *et al*., 2010). Much of Mars' surface is composed of igneous rocks similar to basalt on Earth (Edwards *et al*., 2008; Bandfield *et al*., 2000). As in terrestrial basalts, a prominent component is Fe(II), present in the minerals olivine and pyroxene and in glass (Hoefen *et al*., 2003; Edwards *et al*., 2008).

The Mars-like terrestrial habitat we have analyzed is the rock-ice interface from lava tube caves, which occur frequently in basalt flows. In this type of habitat on Mars, a film of liquid water can exist at the rock's surface, where life is protected from intense solar irradiation. Because it is exposed to the atmosphere, this habitat also has the benefit of an abundant source of energy in the form of redox disequilibrium between the oxidized surface of Mars and Fe(II)-bearing minerals such as olivine and pyroxene. Although iron oxidation can also occur by phototrophy, the most common process to extract energy from Fe(II) minerals on Earth is with oxidants such as dioxygen (O_2) and nitrate (NO_3^-), (Widdel *et al*., 1993; Kappler and Newman, 2004; Schippers *et al*., 2005; Miot *et al*., 2009; Newman, 2010). On Mars, electron acceptors for Fe(II) include putative superoxides and NO_3^- from rock surfaces and atmospheric O_2 (80-130 μbars).

Microbes can influence (trigger or limit) the dissolution of olivines, pyroxene or basalts (Santelli *et al*., 2001; Welch and Banfield, 2002; Benzerara *et al*., 2004; Josef *et al*., 2007; Wu *et al*., 2007). Weathering features and chemical signatures that are

indicative of life were reported in olivine from earth, and similar features were also observed in Mars meteorites (Fisk *et al*., 2006). We proposed that some of these features are produced by neutrophilic iron oxidizing microorganisms using Fe(II) from olivine (Fisk *et al*., 2006). Neutrophilic iron-oxidizing bacteria (nFeOB) are common in freshwater ecosystems (Straub *et al*., 1996; 2004) and marine basalts (Stevens, 1997; Emerson and Moyer, 2002; Edwards *et al*., 2003a,b; Lehman *et al*., 2004; Bailey *et al*., 2009). The most recognized phylotypes belong to the genera *Gallionella*, *Lepthotrix*, *Sideroxydans*, *Marinobacter*, *Mariprofundus* and *Sphaerotilus*. Although best studied in bacteria, this physiotype is also present in some archaea such as *Ferroglobus placidus* (Hafenbradl *et al*., 1996). Recently, a diverse collection of α-, γ- and ζ-Proteobacteria were found that are capable of such activity, although they are not closely related to any previously known nFeOB (Edwards *et al*., 2004; Emerson and Floyd, 2005; Duckworth *et al*., 2009; Wang *et al*., 2009). Even genera that are dominated by heterotrophic species, such as *Pseudomonas* or *Acidovorax*, contain strains that are facultatively or even obligately nFeOB (Kappler *et al*., 2005; Bailey *et al*., 2009). nFeOB may play significant role in the ecology and biogeochemical cycles of basalt-hosted subsurface ecosystems. In a recent paper we reported that bacteria from a basalt subseafloor habitat (Juan de Fuca Ridge) preferentially colonize olivine above all other igneous minerals, and also that many heterotrophic oligotrophic isolates colonizing basalt minerals and glass are facultative nFeOB (Smith *et al.*, 2011).

Olivine ($(Mg,Fe)_2SiO_4$) is a class of minerals that is highly variable regarding the ratio between iron and magnesium. The abundance of iron relative to magnesium

([Fe/(Fe+Mg)]·100) ranges from 0 % Fe(II) in forsterite to 100 % (FeII) in fayalite. Most commonly, olivine crystals contain about 10 % Fe(II). Although olivine only contains iron in reduced form Fe(II), no strain of nFeOB has ever been reported to be capable of using this mineral as a source of energy. Such a finding would be invaluable for studying olivine bio-weathering, for identifying biosignatures and microfossils, for ascertaining the types of life and microhabitats that are present (or were present) on Mars, and for searching for extraterrestrial life. Here we report that olivine-oxidizing nFeOB are present in basalt in cold, near surface, aphotic environments such as caves (*esp.* lava tubes) with permanent ice. The similarity of this environment to environments from Mars suggests that nFeO microorganisms living at the basalt-ice interface could survive on Mars, or may have thrived on Mars in the past when the temperature, atmospheric pressure, and (possibly) the O_2 partial pressure (P_{O2}) were higher than they are today.

Materials and methods

We collected ice and rock fragments from the rock/ice interface in South Ice Cave in the Oregon Cascades (Lat 43^o 34' 59''N, Long 121^o 04' 38''W). South Ice Cave, a basalt lava tube at an elevation of 1530 m., is the result of an eruption on the southern flank of Newberry Caldera, and contains permanent ice. This basalt flow contains ~9.0 % iron as FeO and its mineralogy is primarily plagioclase feldspar, pyroxene and olivine (personal communication Julie Donnelly-Nolan).

The rock/ice samples were stored in sterile bags and packed on ice for transportation to the lab. Culture media were inoculated with melted ice and rock fragments within two

days of collection. Our strategy for isolating olivine-using microbes was to: 1st) produce nFeO enrichments in olivine-containing media; 2nd) isolate colonies of organotrophic oligotrophs on solid media; and 3rd) screen isolates for the capacity to grow on a mineral medium with olivine as the sole source of energy and bicarbonate (HCO_3^-) as the sole source of carbon. For enrichments we used test tubes with 5 mL sterile 0.2 micron-filtered cave water and olivine sand. These enrichment tubes were incubated at 5°C for about 4 weeks to promote the growth of psychrophilic or psychrotolerant microorganisms. The enrichments were inoculated by streaking on R2A organotrophic oligotrophic plates. Colonies exhibiting differing morphologies were saved in a library and then in 50 % sterile glycerol at -80°C.

The mineral medium used for culturing isolates contained per L, 1 mL trace minerals solution, 1 mL vitamins mix, 10 mmol HCO_3^-, 30 mmol phosphate buffer (pH 7), 30 mmol sodium nitrate and 100 g olivine. The trace minerals solution contained: 6.72 mM Na_2EDTA; 5.6 mM H_3BO_3; 1 mM NaCl; 0.54 mM $FeSO_4$; 0.5 mM $CoCl_2$; 0.5 mM $NiSO_4$; 0.39 mM Na_2MoO_4; 0.15 mM $NaSeO_4$; 0.13 mM $MnCl_2$; 0.13 mM $ZnCl_2$ and 0.02 mM $CuCl_2$. The vitamins mix contained per ml: 5 μg p-aminobenzoic acid; 5 μg biotin; 5 μg cyanocobalamin; 5 μg folic acid; 100 μg i-inositol; 100 μg nicotinic acid; 100 μg pyridoxine; 100 μg panthotenic acid; 100 μg riboflavin and 1 μg thiamine. All chemicals were reagent grade. Olivine sand containing 91 % forsterite (Fo_{91}), 0.3-0.8 mm grain size, 8 wt % FeO, was provided by Unimin Corporation. Tumbled crystals of olivine (Fo_{90} beads 1-3 mm in size, ~24 grains/g) were obtained from a local minerals and gems supplier. For most experiments, the olivine was washed with dH_2O to remove

soluble organics and autoclaved in the culture medium. The vitamins mix was added filter sterilized after autoclaving.

Olivine free of organic contaminants was produced by heating it in a furnace at 500 ^{o}C for 90 min in air. After cooling, the olivine showed evidence of surface oxidation (uneven small patches with yellow-rusty appearance). Part of the iron oxides were removed by acid dissolution in three 24 hrs. long washes with occasional stirring at room temperature. The acid washing solution contained 0.25 ml/L H_2SO_4 and 20 mM Na_2SO_4, pH ~2.5, and was used in a proportion of about 10 g olivine to 100 ml solution. We compared the UV spectra of the various washes with the spectra of control unheated olivine and calculated the concentration of Fe(III) relative to a standard. Under these conditions ferric iron absorbs strongly in the 295-304 nm range, while ferrous iron absorbs mostly in the 220-250 nm range (Steiner and Lazaroff, 1974). In this method 304 nm peaks in solutions containing Fe(II) are used as evidence of contamination with Fe(III). This method allows detecting concentrations of Fe(III) as low as 20 μM even in the presence of high concentrations of Fe(II) because the absorbance of Fe(III) at 304 nm is ~300 times larger than that of Fe(II). After the acid treatment the olivine was washed with dH_2O and dried in a 55 ^{o}C oven. Macroscopically, the heated olivine retained a yellow-green appearance with pink-rusty patches. Under a dissecting scope most olivine sand particles appeared transparent and colorless, while olivine beads appeared pale green and translucent.

For phylogenetic identification we obtained biomass by growing cells in liquid TSB medium in aerobic conditions. Cells were separated by centrifugation (14,000 rpm at 2

^{o}C for 5 min.), genomic DNA (gDNA) was extracted with a Qiagen genomic tip kit, and quantified with a NanoDrop1000 instrument. A fragment of the SSU rRNA gene was amplified by PCR using the primers 8F (5'- AGAGTTTGATCCTGGCTCAG) and 1492R (5'- GGTTACCTTGTTACGACTT) (Lane, 1991; Baker *et al*., 2003). We used 20 µL PCR volumes containing 10 µL Fermentas mix, 0.8 µL of each µM primer, 6.5 µL of dH_2O and 2 µL of 100 ng/µL gDNA. The PCR conditions were: denaturing at 95 ^{o}C for 5 min.; 40 cycles of 94 ^{o}C for 30 sec., 50 ^{o}C for 30 sec. and 72 ^{o}C for 2 min.; and final extension at 72 ^{o}C for 7 min. The size of the PCR products was verified by 0.7 % agarose electrophoresis, and the remaining 15 µL of the PCR products were cleaned with an UltraClean PCR DNA purification kit (MoBio). The amplicons were sequenced at the DNA Sequencing Core facility of Oregon Health Sciences University with three primers: 8F, 515F (5'-GTGCCAGCMGCCGCGGTAA) and 1492R (Baker *et al*., 2003) by capillary electrophoresis on an ABI 3130xl instrument. Duplicate sequences were manually aligned, and when differences between duplicates were found we repeated the PCR and sequencing to compare triplicates for each sequence. The sequences of each isolate were assembled into contiguous DNA fragments and blasted in the Ribosomal Database for phylogenetic identification. The sequence of *Pseudomonas sp.* HerB (a total of 1362 positions) was imported into MEGA 4 (Tamura *et al*., 2007) and aligned with phylogenetic relatives. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to

the branches (Felsenstein, 1985). The evolutionary distances (base substitutions per site) were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004).

To characterize the O_2 preference of the isolates, we inoculated TSB gradient tubes with 0.15 % agar and 2 mg resazurin per L and growth was observed. The capacity of the isolates to grow as microaerophilic iron oxidizers was verified in gradient tubes with semisolid medium (0.15 % agar), and 2 % agar plug containing either 36 mM $FeCO_3$ or olivine sand as the Fe(II) source, (modified after: Emerson and Moyer, 1997; Emerson and Floyd, 2005). After growth was seen in a gradient tube we repeated inoculation a couple of times from tubes with growth to fresh tubes using a stabbing needle. Growth by iron oxidation was also verified in serum bottles with liquid mineral medium with 5 mM soluble ferrous sulfate, at pH 7 and under 1.6 % O_2. When testing for nitrate reduction capabilities, we used a medium with 10 mM NO_3^- at pH 7 (DIFCO Catalog #226810) in culture tubes containing an inverted Durham tube to capture N_2 gas that may be produced by denitrification. After 5 days of incubation the cultures were examined for evidence of denitrification, and tested for nitrate and nitrite reduction (Leboffe and Pierce, 2010). To verify growth by olivine oxidation, we incubated cells in test tubes with mineral medium with or without olivine. Growth on TSB organotrophic medium was monitored by spectrophotometry (Abs600) and microscopy. Media with olivine sand contain suspended mineral particles making spectrophotometric data difficult to interpret. Therefore, growth on olivine-containing media was determined by microscopy. When sufficient cells were present in a suspension $>10^4$ cells/ml we counted sufficient fields to account a total of 100-300 cells. To test for autotrophic growth while in olivine-

containing mineral media, we incubated the cells with various concentrations of HCO_3^- and no other sources of carbon. Incubations in liquids under microaerophilic conditions were done in serum bottles sealed with a 1 cm thick butyl stopper and purged prior to autoclaving with dinitrogen gas containing 1.6 % O_2. The O_2 concentration in the head space was measured by gas chromatography (SRI 310C instrument, Molecular sieve column and TCD detector). The gas pressure was measured with an Omega pressure meter (Omega Engineering, Inc. CT). The concentration of O_2 in the liquid phase was derived from a saturation of 236 μM O_2 in freshwater with air at 760 mmHg and at 30 ^oC.

Results

Of all aerobic heterotrophs that we have isolated eleven strains were also capable of growing in a mineral medium with olivine as the sole source of energy. Seven of these strains were mesophilic γ-*Proteobacteria* from the genus *Pseudomonas*, two strains were cryophilic *Brevundimonads* (α-*Proteobacteria*), and two strains (also cryophilic) belong to the genus *Acidovorax* (β-*Proteobacteria*). One strain, *Pseudomonas sp*. HerB, was selected for further work because among all isolates it reached the highest density while growing in a mineral medium with 10 % w:v olivine sand, 20 mM HCO_3^-, 1 mL vitamins mix per L, pH 7 and 30 ^oC. Starting from ~10^3 cells/ml this strain reached ~$5 \cdot 10^7$ cells/ml in one week. It's position in a phylogenetic tree and a microscopic image of cells are shown in Fig. 1.

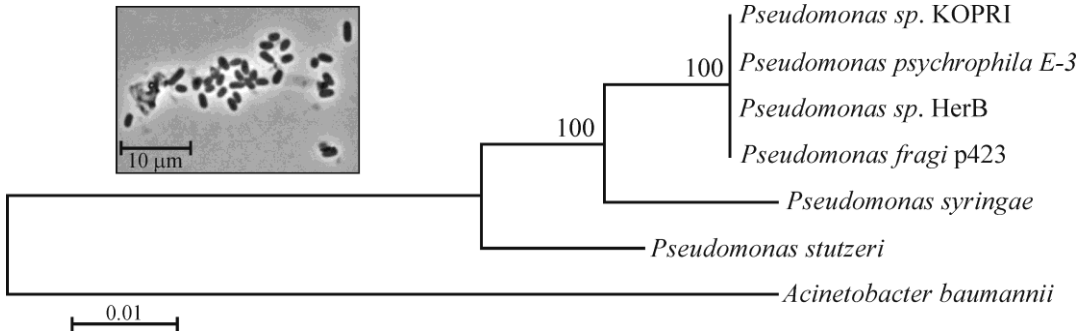

Fig. 1. Tree indicating the phylogenetic position of *Pseudomonas sp*. HerB. The insert shows cells of *Pseudomonas sp*. HerB grown in R2A medium seen by phase contrast optical microscopy at 1000x. The same cell shape and size was seen when HerB cells grew in $FeCO_3$ gradient tubes and in olivine-containing liquid mineral medium.

We analyzed this strain for: the ability to oxidize soluble Fe(II) and olivine Fe(II); the capacity to respire O_2 and NO_3^-; the effect of pH and HCO_3^- on growth, O_2 concentration preference in a olivine containing mineral medium; minimum, maximum, and optimum temperatures for growth; growth in vitamins-limited mineral media with olivine as the sole source of energy and HCO_3^- as a carbon source, and growth in mineral medium with olivine freed of organic contaminants.

Cells of *Pseudomonas sp*. HerB are aerobic under heterotrophic conditions. Growth was faster on TSA plates and TSB tubes in air than with 1.6 % O_2. In mineral media with olivine sand as the source of energy and at pH 7, growth was very slow under 21 % O_2, better at ~5 % O_2, and best at ~1.6 % O_2 where cultures reached densities of ~$3 \cdot 10^7$ cells/mL after seven days of incubation. Figure 2(a) shows the effect of olivine on the growth of *Pseudomonas sp*. HerB in mineral medium (with/without olivine, and with/without NO_3^-). No growth occurred when olivine was absent, but in the presence of olivine the culture reached $2.5 \cdot 10^7$ cells/mL without NO_3^- and $3.7 \cdot 10^7$ cells/mL with

NO_3^-. The difference between with/without NO_3^- was within one standard deviation based on triplicates, and thus statistical difference between these treatments could not be confirmed. Independent measurements showed no NO_3^- reducing capability by this strain. We also verified that cells of *Pseudomonas sp*. HerB consume O_2 while growing in a mineral medium with olivine (Fig. 2(b)) and estimate that a 10 mL culture used 180 μmol of O_2 in 20 days. According to Rxn. 2 (from the Discussion section) and the iron content of the olivine sand we have used this O_2 is equivalent to dissolving ~290 mg olivine.

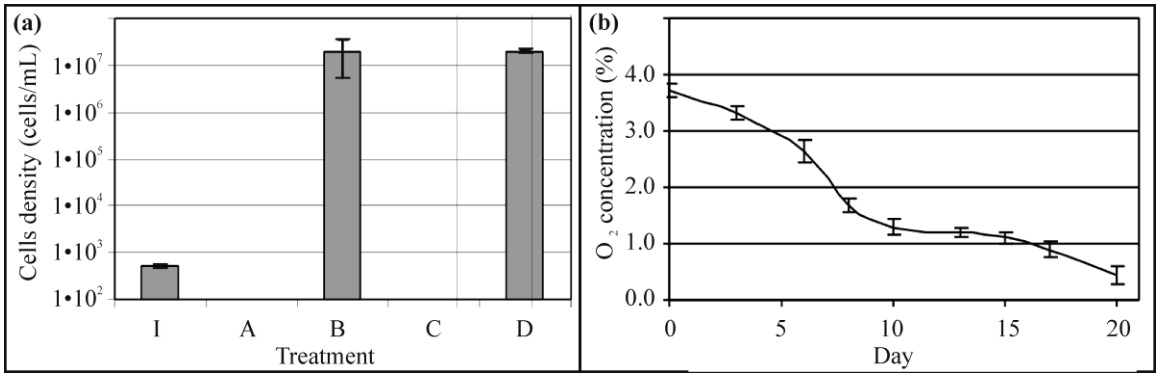

Fig. 2. (a) The growth of *Pseudomonas sp*. HerB with olivine as a source of energy. Incubation in Hungate tubes with 10 mL medium, 20 mM HCO_3^-, 1 mL/L vitamins mix, pH 7, 1.6 % O_2 at 30°C. In this experiment we compared growth with/without 10 % w:v olivine sand, and with/without 10 mM NO_3^-. I = Initial cell density (~$7.7 \bullet 10^2$ cells/mL). A = No olivine, no NO_3^-. B = with olivine, no NO_3^-. C = No olivine, with NO_3^-. D = with olivine, with NO_3^-. Cell counts are based on triplicates and were determined by microscopy after seven days of incubation. Error bars are one standard deviation. (b) Evolution of the O_2 concentration in gas phase during the growth of *Pseudomonas sp*. HerB in a mineral medium with olivine (subtracted from an un-inoculated control). Incubations were in 140 mL serum bottles with 10 mL mineral medium, 10 % w:v olivine sand, 20 mM HCO_3^-, 1 mL/L vitamins mix, pH 7, and ~1.1 bar initial pressure at 30°C. The error bars are standard deviations based on triplicate readings of one bottle.

The ability of *Pseudomonas sp*. HerB to grow as a neutrophilic iron oxidizer was independently verified in gradient tubes with iron carbonate in a bottom agar plug, and in liquid mineral medium with 5 mM Fe(II) at pH 7 and under 1.6 % O_2 (images not shown). Growth was also seen when gradient tubes were inoculated from gradient tubes into other gradient tubes. We also compared growth on a mineral medium with 10 % w:v olivine sand relative to growth in the same mineral medium with 5 mM soluble Fe(II) initial concentration, 20 mM HCO_3^-, pH 7, 1.6 % O_2, and incubate at 30 ^oC. The extent of growth was similar between these two media. The cultures containing olivine reached ~$8 \bullet 10^6$ cells/mL in 10 days. Because the autooxidation of soluble iron is rapid in neutral to alkaline conditions, the available Fe(II) in solution is low at pH 7, limiting the growth of nFeOB.

We verified the growth of *Pseudomonas sp*. HerB in mineral medium containing olivine at six pH values (Fig. 3(a)). No growth was observed at pH 4.5 and pH 8.5, and the largest cell density (after one week of incubation), was seen at pH 7. In environments rich in carbonate and poor in phosphate, bicarbonate may precipitate the Fe(II) from olivine, and the surface of olivine may weather faster or become coated with a thin layer of ferrous carbonate (such as siderite). Less is known about this process than about the formation of magnesite from bicarbonate-exposed olivine (Giammar *et al*., 2005; Andreani *et al*., 2009). For this reason HCO_3^- may also influence the growth of olivine-oxidizing nFeOB. Furthermore, in the mineral oligotrophic media we have used, HCO_3^- and CO_2(g) are the only sources of carbon, and thus only strains that are also facultative autotroph can grow. Fig. 3(b) shows the effect of HCO_3^- on the growth of *Pseudomonas*

sp. HerB in mineral media containing olivine. No growth was observed without HCO_3^-, ~$1 \cdot 10^7$ cells/mL at 10 mM HCO_3^- and little variation in cell density above 10 mM HCO_3^-. In this experiment we also incubated cells in controls without olivine and (similar to above) no measurable growth was seen.

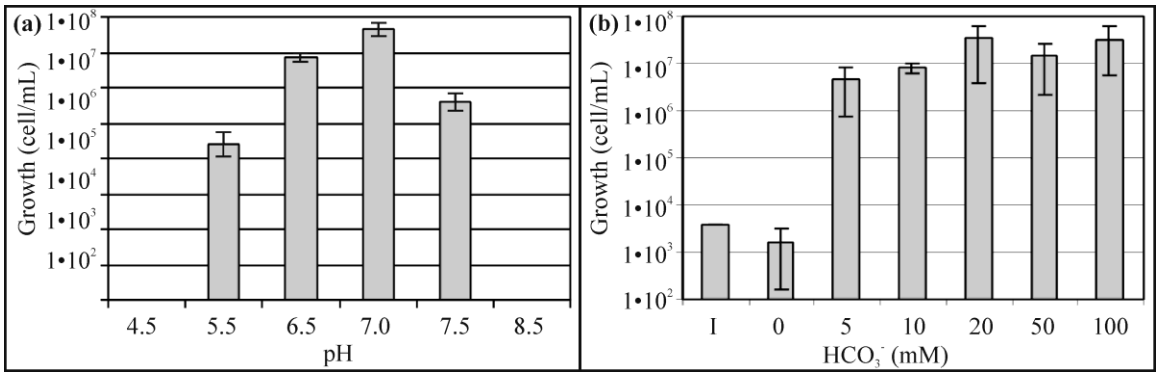

Fig. 3. (a) The growth of *Pseudomonas sp*. HerB in olivine-containing mineral media at different pHs. The media contained 20 mM HCO_3^-, 10 % w:v olivine sand and 1 mL/L vitamins mix, and incubations occurred under 1.6 % O_2 at 30°C for seven days. (b) Growth in the same mineral medium at pH 7 and with various concentrations of HCO_3^-, incubated for 14 days at 20 °C. I = Initial cell density (~$3.8 \cdot 10^3$ cells/mL). The error bars are one standard deviation from triplicates.

To study the effect of temperature on growth we analyzed it at 2 °C, 5 °C, 10 °C, 15 °C, 25 °C, 30 °C, 37 °C and 40 °C. We compared the growth rates at different temperatures using the slopes of the exponential growth phases (Fig. 4(a) and (b)). We found the following cardinal temperatures for olivine growth: ~4-5°C minimum, ~12-14°C optimum and ~30-31°C maximum (Fig. 4(b)).

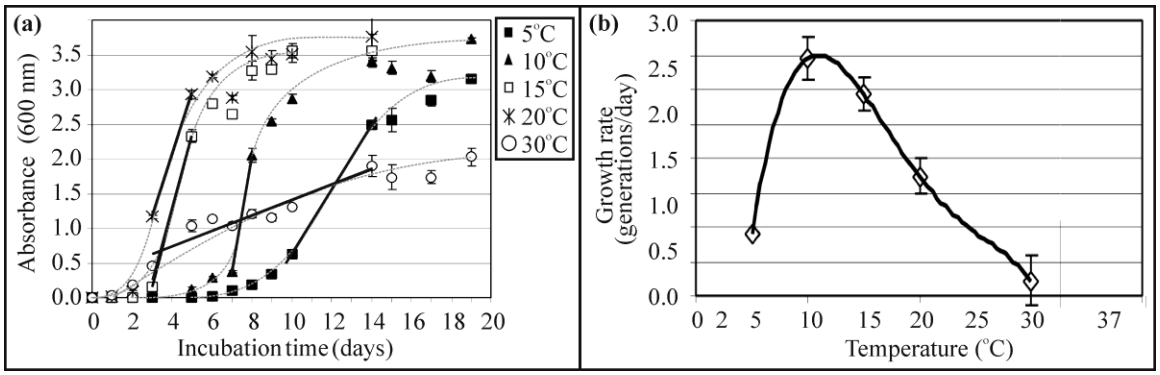

Fig. 4. (a) Growth profiles of *Pseudomonas sp*. HerB in TSB medium at five temperatures (5°C; 10°C; 15°C; 25°C and 30°C). No growth was seen at 2°C, 37°C and 40°C. The values shown are averages of triplicates and the errors bars equal 1SD. The interrupted lines are hand drawn and help observe the general trend of each set of data. The straight lines are linear regression slopes for the data points situated near and opposite sides of the inflexion point of the polynomial fit, in the part of the curve that represents the exponential growth phase. All cultures started from ~10^3 cells mL^{-1} and were incubated in 18 mm diameter test tubes with 10 mL medium. The error bars are 1 SD from triplicates. (b) The effect of temperature on the growth rate, calculated based on the slope of the exponential phase shown in (a). The error bars from (b) are 1SD of the expected variation in the slope of exponential growth in (a) based on +/-1SD of cell density.

Because Pseudomonads can metabolize a wide variety of organic molecules, excess of vitamins in a mineral medium may represent additional sources of carbon and energy. Fig. 5 shows the growth of *Pseudomonas sp*. HerB in mineral media with olivine and various concentrations of vitamins. In the olivine-containing media good growth (>10^7 cells/mL) was seen in all treatments. In the olivine-absent media growth was not observed when the vitamins mix was ≤ 1 mL/L.

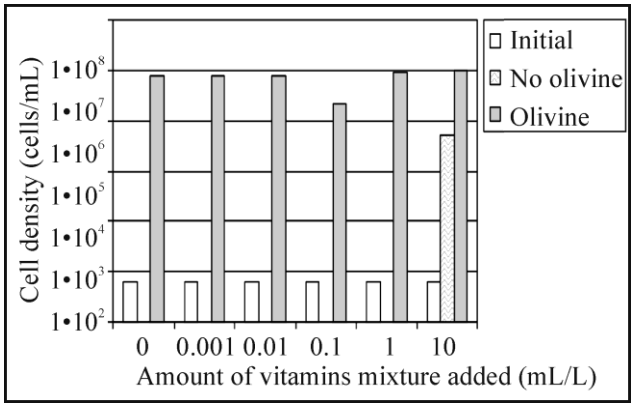

Fig. 5. Growth of *Pseudomonas sp*. HerB in mineral medium (with/without olivine) and various abundances of vitamins mix. Incubations occurred in test tubes with 5 mL mineral medium, 10 mM HCO_3^-, with/without 20 olivine beads (~840 mg olivine per tube), and at pH 7. All cultures started from ~$5.3 \cdot 10^3$ cells/mL. The graph shows cell densities after 7 days at 1.6 % O_2 and 20°C.

To determine whether the olivine surface is a limiting factor in the growth of *Pseudomonas sp*. HerB we compared growth in the presence of olivine sand *vs*. olivine beads. In these experiments we also compared growth at 5°C *vs.* 20°C (Fig. 6). We expected finding higher cell density with olivine sand than beads and at 20°C than at 5°C. Olivine sand with 0.4 mm particle diameter has a calculated surface area of ~7,000 mm^2/g, while olivine beads with 3 mm diameter have about 120 mm^2/g (*i.e*., an about 55 fold decrease in the surface surface:mass ratio). After seven days of incubation we found about two fold increase in cell density in sand ($4.6 \cdot 10^7$ cells/mL) *vs*. beads ($2.1 \cdot 10^7$ cells/mL), and no significant differences in cell density between 5°C and 20°C treatments.

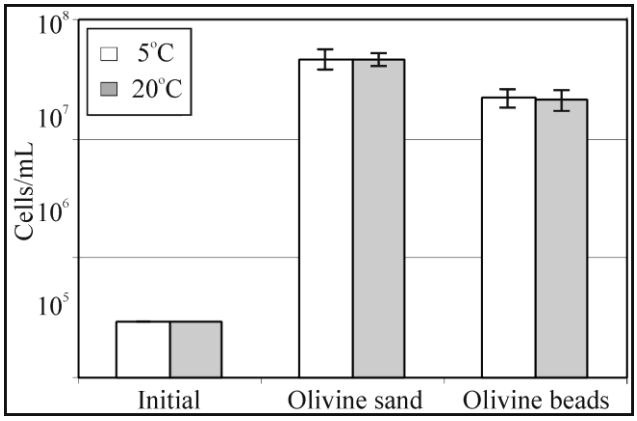

Fig. 6. The growth of *Pseudomonas sp*. HerB in mineral medium with olivine of two particle sizes (olivine sand and olivine beads) and at two temperatures (5°C and 20°C). Incubations were for seven days in Hungate tubes, 5 mL mineral medium, 10 % w:v olivine, 20 mM HCO_3^-, pH 7, 1.6 % O_2, and 1 mL/L vitamins mix. The error bars are +/- 1 SD from triplicates.

We verified whether the growth of growth of *Pseudomonas* sp. HerB on olivine may be explained by organics contaminants. In this experiment we used liquid mineral medium (shown above), with 20 mM HCO_3^-, 0 mM nitrate, pH 7.0, 5 ml medium in Hungate tubes, ~1 g olivine per tube, sealed and crimped, purged with 1.6 % O_2 and autoclaved. A volume of diluted vitamins mixture solution was injected filter sterilized after autoclavation to a final concentration of 1 ml/L. We inoculated washed cell pellets, from serial dilutions into tubes containing heat-treated vs. non-heat treated olivine and sand vs. beads as well as medium without olivine. The initial cell density, calculated based on cell density in the serial dilutions, was ~$3 \cdot 10^2$ cells/ml. We found that *Pseudomonas* sp. HerB grows in olivine freed of organics, and no significant differences (within 1 SD) between the heat treated and non-heat-treated olivine (Fig. 7).

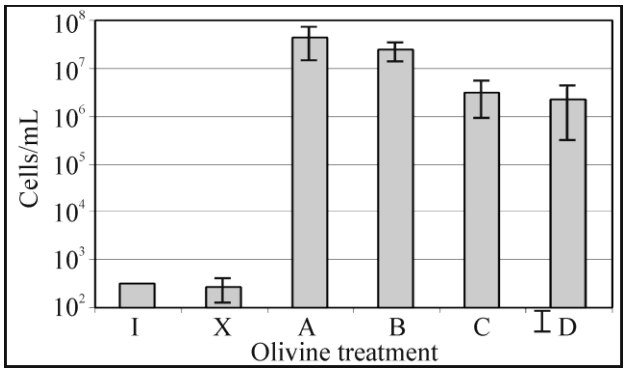

Fig. 7. The growth of *Pseudomonas sp*. HerB in mineral medium with olivine sand and olivine beads after removing traces of organics by heating the crystals at 500^{o}C. Incubations occurred for 14 days in Hungate tubes, with 5 mL mineral medium, 10 % w:v olivine, 20 mM HCO_3^-, pH 7, 1 ml/L vitamins mix and 1.6 % O_2. The treatments shown in the graph are: I = initial cell density; X = no olivine present; A = non heat treated olivine sand; B = heat-treated olivine sand; C = non-heat-treated olivine beads; and D = heat-treated olivine beads. The values shown are averages of triplicates and error bars equal 1 SD.

Discussion

nFeOB have been reported to inhabit seawater, freshwater, groundwater, terrestrial basalts, subseafloor, hydrothermal systems, iron oxyhydroxide mats, and the surface of glass and Fe(II)-containing minerals from a wide variety of sources (Emerson and Moyer, 2002; Kappler *et al*., 2005; Edwards *et al*., 2003a;b; Gronstal *et al*., 2009; Miot *et al*., 2009). Our finding extends the palette of environments where nFeOB exist to the basalt/ice boundary habitat in a lava-tube ice cave were olivine is present. The properties of this habitat (near 0 ^{o}C, dark, oligotrophic, circumneutral pH, and at the interface between basalt and ice near an oxidized atmosphere), makes it a terrestrial analogue for a near-surface aphotic environment on Mars, where life may exist today, or could have thrived in the past when the atmospheric pressure and surface temperature of Mars were higher than today.

Of eleven strains of nFeOB we have isolated, we report the physiology of one strain, *Pseudomonas sp*. HerB. Regarding the source of carbon, this microorganism is heterotroph and facultative autotroph (capable of using CO_2 as a sole source of carbon). Regarding the source of energy, this strain is an organotroph facultative chemolithotroph, capable of neutrophilic iron oxidation with Fe(II) from olivine as the electron donor and O_2 as the electron acceptor. The optimum pH for growth by olivine oxidation was ~7, but we did not verify to what extent this optimum is related to competition with iron self-oxidation or to metabolic pH preference by this strain. Regarding temperature preference, *Pseudomonas sp*. HerB is sub-mesophilic. The optimum growth (measured in TSB medium) was about 14-15^{o}C, but cells grew at temperatures as low as 5^{o}C. Based on the growth profile we predict that the minimum temperature for growth is ~4^{o}C. Regarding O_2 tolerance, this strain is microaerophilic and facultative aerobe, and the growth in olivine-containing mineral medium is faster at low O_2 concentration (1.6 %) than at higher O_2 concentrations (5 % and 21 %). The fact that most of our isolates are from the genus *Pseudomonas* is not unexpected. Pseudomonads are versatile and show all the metabolic capabilities described above. Two strains of *Pseudomonas* were shown to be neutrophilic iron oxidizers (Bailey *et al*., 2009). Pseudomonads are important denitrifiers in soil (Chan *et al*., 1994; Smil, 2000), but *Pseudomonas sp*. HerB strain is not a denitrifier and cannot reduce nitrate. The pathway for carbon fixation in *Pseudomonas sp*. HerB is unknown, yet some Pseudomonads were shown to be facultative autotrophs fixing CO_2 via RuBisCo (Mahmood *et al*., 2009; Morikawa and Imanaka, 1993; Yuliar, 1997).

The mechanism of dissolution of olivine in the presence of *Pseudomonas sp*. HerB is unclear. If a specialized means to extract Fe(II) from the olivine crystals does not exist then the growth of this olivine-using nFeOB should be controlled predominantly by the rate of olivine dissolution and by the kinetics of chemical Fe(II) oxidation. The growth of olivine-oxidizing nFeOB may be favored by low temperature, low O_2, and the presence of HCO_3^- or other iron-binding agents. Low temperature and low O_2 help decrease the kinetics of iron oxidation, increasing the availability of soluble Fe(II). The role of low temperatures in controlling the growth of nFeOB is little studied. The fact that nFeOB prefer low O_2 conditions is well known; it is due to competition between microbial iron oxidation and chemical oxidation (Edwards *et al*., 2003b). HCO_3^- is one of the abiotic factors that may contribute to olivine bioweathering by binding Fe(II) and forming siderite, which is easier to oxidize than olivine. nFeOB are already known to use Fe(II) carbonate as a source of energy (Emerson and Moyer, 1997; Emerson and Floyd, 2005).

The pH may also play an important role in the oxidation of olivine Fe(II). The redox potential (E^o) of the Fe^{3+}/Fe^{2+} couple is pH-dependent and takes more positive values in acidic conditions (Thauer *et al*., 1977). Hence, iron oxidation is more exergonic at neutral pH than at acidic pH.

$Fe^{2+} + ½O_2 + 2H^+ \rightarrow Fe^{3+} + H_2O$ Rxn.1

at pH 2 the E^o of Fe^{3+}/Fe^{2+} = + 0.77 V and ΔG^o = - 8.7 kJ per mol Fe

$Fe^{2+} + ½O_2 + 2H_2O \rightarrow Fe^{3+}(OH)_3 + H^+$ Rxn.2

at pH 7 the E^o of Fe^{3+}/Fe^{2+} = + 0.20 V and ΔG^o = - 63.7 kJ per mol Fe

The dissolution of olivine in water at low O_2 partial pressure and in the presence of carbonate leads to magnesite and siderite. The formation of magnesite is favorable at pH 3-6; 30-90^{o}C and PCO_2 between 1 and 100 bar (Giammar *et al*., 2005; Andreani *et al*., 2009) and was extensively studied (Goff and Lackner, 1998; Guthrie *et al*., 2001; Schulze *et al*., 2004; Giammar *et al*., 2005; Andreani *et al*., 2009).

$$MgSiO_4 + 2CO_2 \rightarrow 2MgCO_3 + SiO_2 \quad \Delta G = -95 \text{ kJ/mol } CO_2 \qquad \text{Rxn.3}$$

The formation of siderite from fayalite has a similar form.

$$Fe_2SiO_4 + 2CO_2 \rightarrow 2Fe(II)CO_3 + SiO_2 \qquad \text{Rxn.4}$$

The carbonation of olivine iron at 11.5-19 MPa and 155-185 ^{o}C is fast, ~50 % per hour (O'Connor *et al*., 2001; Hanchen *et al*., 2006). The rate of olivine carbonation at low temperatures, and the participation of iron in this carbonation have been little studied. After ferrous carbonate has been formed, its oxidation is exergonic and can be used as an energy source by nFeOB. Our results indicate that *Pseudomonas sp*. HerB is capable of such activity.

The oxidation of iron on the surface of olivine is inhibited by magnetite and silica-rich layers (Andreani *et al*., 2009), and also by Fe(III) oxide-rich layers (Santelli *et al*., 2001; Welch and Banfield, 2002; Josef *et al*., 2007). In early stages of olivine oxidation in the presence of HCO_3^-, iron oxidation by the cells may be out-competed by abiotic oxidation and carbonation, but after ferrous carbonate has been formed, biological iron-oxidation is probably fast. If this mechanism is correct, then O_2, pH, and HCO_3^- should be important controllers of the kinetics of olivine bio-dissolution by nFeO microorganisms. The abundance of Mg in olivine is another limiting factor of this

process, because magnesite layers formed on the surface of olivine should inhibit further olivine dissolution. The removal of Mg from olivine at a rate higher than the chemical weathering requires energy. Because Mg is not coupled to any redox reaction, we hypothesize that increase in the concentration of Mg in olivine will have negative effect on the rate of biodissolution via nFeOB.

Conclusions

We report for the first time that a strain of nFeOB from the genus *Pseudomonas* is able to grow by using the mineral olivine as a source of energy. We propose that such microbes are common in nature, and that their microenvironment has to satisfy some specific requirements. Some of the most important are circumneutral pH, low P_{O2} and low temperature. On Earth such conditions can be encountered at basalt-ice interfaces where liquid water is also present. This finding is important for astrobiology because the environmental conditions in the recent geological past of Mars (higher pressure and temperature than today) would have allowed such microbes to thrive near the surface in lava tubes, under the ice sheet, and in the basalt subsurface where cells are protected from harmful UV radiation, yet still benefit from the oxidants from Mars's surface. Orbital and surface observations of Mars confirmed that igneous rocks are exposed over significant areas (Edwards *et al*., 2008; Bandfield *et al*., 2000), and that some areas are dominated by olivine-bearing rocks (Hoefen *et al*., 2003; Edwards *et al*., 2008). In addition, skylights interpreted as entrances to lava tubes (a physical environment similar to South Ice Cave), have been observed on the flanks of Martian volcanoes (Cushing *et*

al., 2007). Martian caves could contain permanent ice (Williams *et al*., 2010) and life in Martian caves has already been proposed to exist (Boston *et al*., 1992).

Calculations of autotrophic energy-producing reactions likely to occur on Mars suggest that the oxidation of Fe(II) by O_2 or NO_3^- could drive microbial ecosystems (Jepson *et al*., 2007). Applying $\Delta G = \Delta G^{o'} + TR\ln Q$ to Rxn.2 (for $T \approx 0$ ^oC) it can be shown that this reaction is exergonic (ΔG = -4.2 kJ/mol) even at P_{O2} = 0.1 mbars. Notably, the P_{O2} on Mars (derived from ~7 mbars total pressure and ~0.13 % O_2), is ~0.9 mbars (Seiff and Kirk, 1977).

A key requirement for Earth-colonizing cellular life (including nFeOB) is the presence of liquid water. Even the present day subsurface and the sub-ice conditions on Mars may harbor such microbes because thin films of water exist in soil, even below freezing (Anderson and Tice, 1973). Low-temperature brines (maintaining liquid water at temperatures as low as -20 ^oC) could have existed over much of Mars's history (Fairen, 2010). Multiple lines of evidence indicate that Mars had liquid water at the surface in the past (Carr, 1995; Haberle *et al*., 2001; Head *et al*., 2003; Carr and Head, 2010; Warner *et al*., 2010). Thus, some areas of the shallow subsurface of past Mars satisfy two requirements for nFeO-based cellular life: liquid water and redox energy in the form of olivine Fe(II) in disequilibrium with oxidized chemicals from the planet's surface. In the event of slight increases in temperature and pressure on the surface of Mars (such as during terraformation activities, orbital forcing, or release of greenhouse gas from buried hydrates), olivine-using nFeO microorganisms would be some of the first colonists and primary producers of the newly-formed Mars ecosystems.

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